

SEROTONIN AND THE RAT ADRENAL GLAND

by

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DECLARATION

This thesis was composed by myself, and the work presented in it is entirely my own. Measurements made by others in collaborative studies and figures used in the text from other sources are acknowledged and indicated in the text.

Soraya Shaikh

November 1987

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DEDICATION

I dedicate this thesis to my parents

ABSTRACT

Serotonin (5HT) is an indoleamine with potent in vitro and in vivo effects on aldosterone biosynthesis in the adrenal zona glomerulosa of several species including Man and the rat. Its physiological role in the control of aldosterone secretion however, is not well understood. The purpose of these studies was to clarify its possible role in aldosterone biosynthesis using the rat as an experimental model.

Attempts to localise 5HT within the adrenal gland using a specific monoclonal antibody to 5HT with the avidin-biotin detection technique, suggested that serotonergic nerves are not present in the adrenal cortex although appreciable levels of 5HT were measured by HPLC in adrenal tissue ($1.7 \pm 0.2 \mu\text{g/g}$ wet weight). The storage and metabolism of 5HT in isolated zona glomerulosa cells and inner cortical and medullary cells was investigated using [^3H]-5HT as a marker. Zona glomerulosa cells rapidly metabolised 5HT, whereas the inner zones were able to store or retain 5HT to a greater extent. Stimulation of steroidogenesis by 5HT however, was confined to the zona glomerulosa cells of the adrenal cortex.

The interaction of 5HT with specific 5HT receptors was investigated by studying the effects of the 5HT antagonists, methysergide and ketanserin on the steroidogenic response to 5HT in isolated zona glomerulosa cell suspensions. Methysergide (10^{-6}M) inhibited the corticosterone and aldosterone responses to 5HT ($10^{-9}\text{M} - 10^{-6}$) and angiotensin II (10^{-9}M , 10^{-8}M). In addition, it significantly

inhibited the corticosterone response to ACTH. Ketanserin (10^{-6}M) also inhibited the corticosterone and aldosterone responses to 5HT (10^{-9}M , 10^{-8}M) and AII (10^{-9}M , 10^{-8}M) but did not affect ACTH-stimulated steroidogenesis. Neither antagonist affected the steroidogenic responses to potassium.

A change in dietary salt intake of rats did not lead to any significant change in either the blood levels or adrenal contents of 5HT. The in vitro-responsiveness of adrenal cells to 5HT however, was altered by changing the sodium status.

An overall view of changing endogenous levels of 5HT in vivo in the rat was explored using the 5HT-depleting agent, PCPA and the immediate 5HT-precursor, 5-hydroxytryptophan (5HTP). Treatment with PCPA led to a decrease in blood 5HT levels and a loss of in vitro responsiveness to 5HT and AII in subsequent preparations of zona glomerulosa cells. Treatment with 5HTP produced higher blood levels of 5HT and an enhanced responsiveness of subsequent preparations of zona glomerulosa cells.

These studies suggest that 5HT from central and/or peripheral sources such as platelets or mast cells, could exert a tonic effect on aldosterone secretion from the rat adrenal zona glomerulosa, which is mediated by the activation of specific receptors for 5HT.

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Chapter 1

PROPERTIES OF SEROTONIN

1.1 INTRODUCTION

Serotonin or 5-hydroxytryptamine (5HT) has been the subject of many investigators since it is a ubiquitous compound. One of the major discoverers of 5HT, Irvine Page commented, "Serotonin in walnuts and bananas and in jelly-fish and in carcinoids and in brain leaves me with a sense of bewilderment and fear that someone will ask me what serotonin does". Rapport et al., (1948) were the first to identify the potent vasoconstricting substance originally called vasotonin or vasoconstrictin in plasma, as 5HT. Earlier studies by Erspammer and coworkers in 1933 (see Erspammer et al., 1952) had also unwittingly revealed the substance in isolated gastric mucosa.

1.2 ORIGIN OF SEROTONIN

Serotonin is widespread throughout the plant and animal kingdom. It is found in high concentrations in bananas, pineapples, tomatoes, strawberries and in some poisonous plant species including the stinging fluid of the common nettle. In the lower animal kingdom it is found in very high concentrations in the molluscs and annelida, particularly in ganglionic structures; in the octopus and the blowfly, 5HT exists in the salivary glands (Berridge 1979), and in both cases it may serve to augment salivary secretions. It is also found in the venom of scorpions, and in the skin of some amphibia e.g. bufo marinus. In mammals, 5HT is localised predominantly in the central nervous system (CNS) and the gastro-intestinal tract (GI). It is also present in the blood and to a lesser extent, 5HT has been localised in endocrine organs such as the pituitary gland in the rat

Fig 1.1 Ionic forms of serotonin

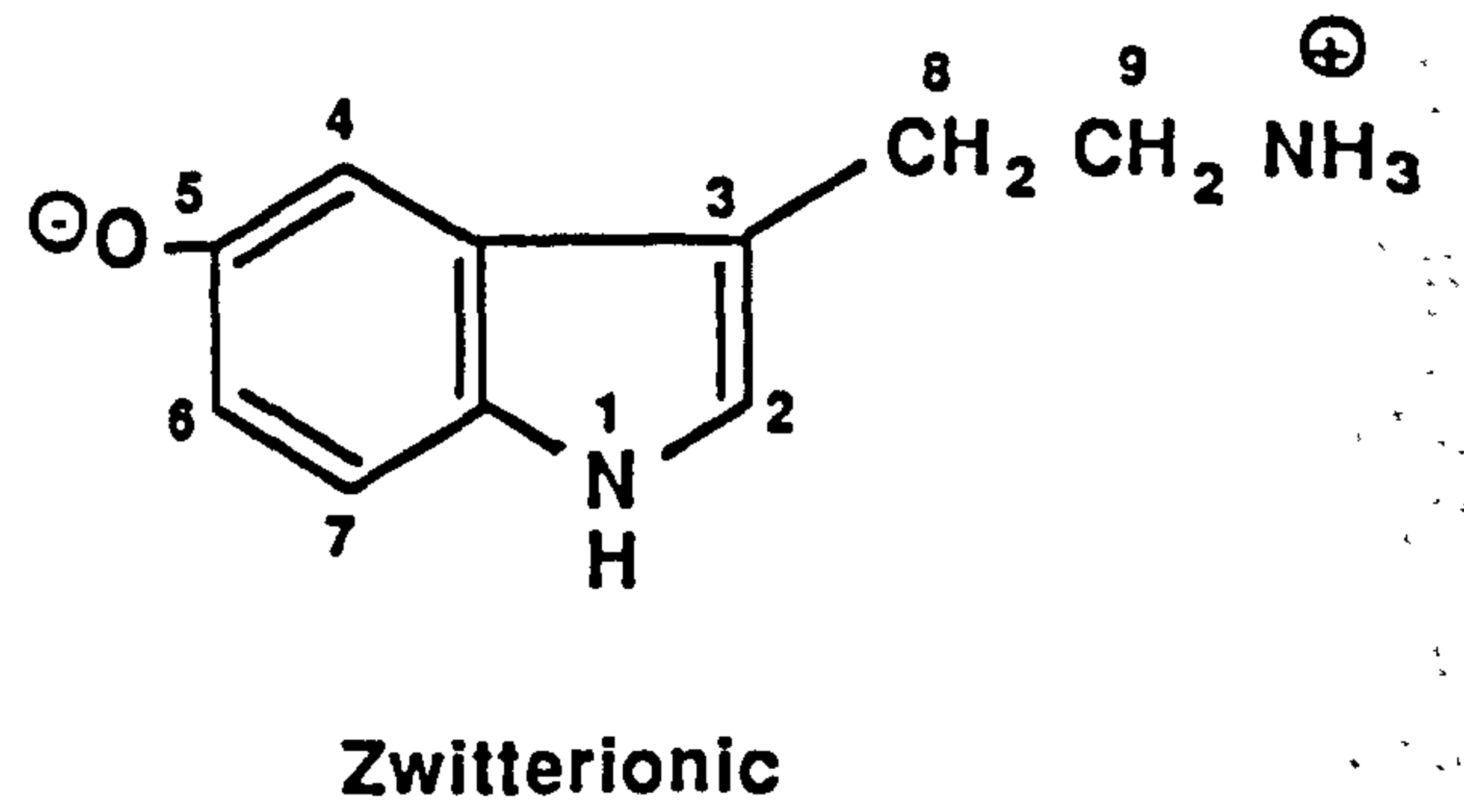
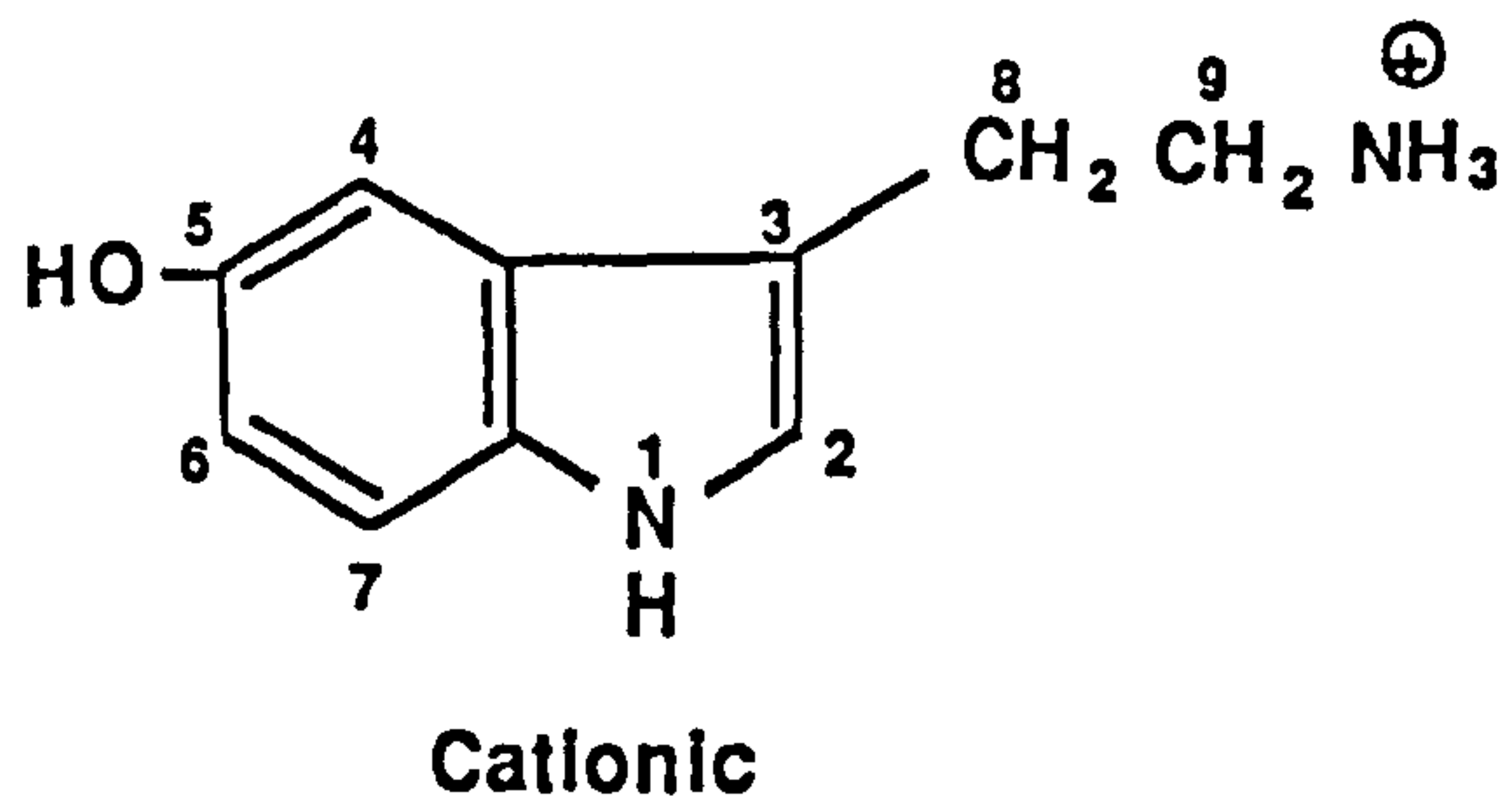
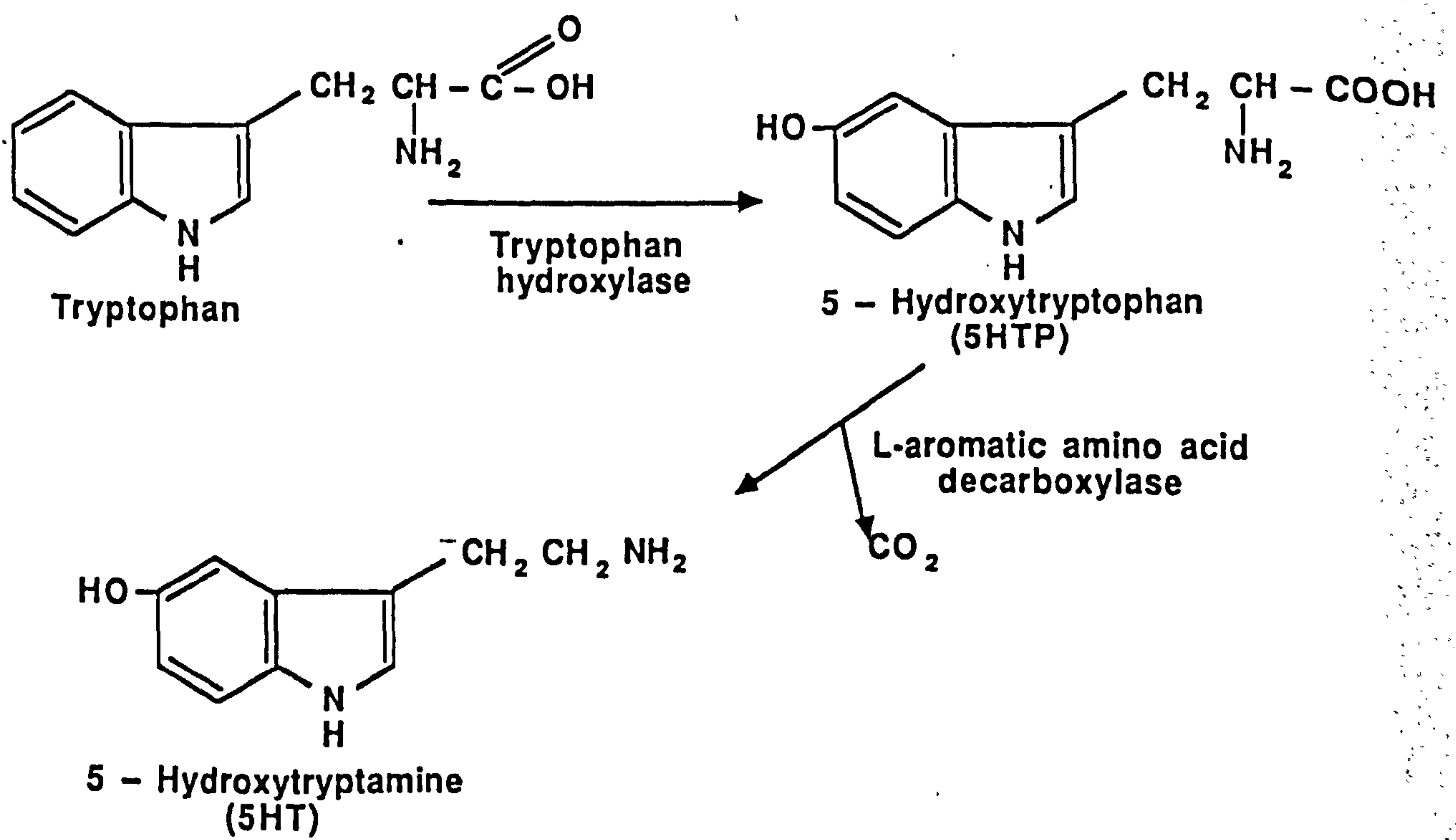


Fig 1.2 Biosynthesis of serotonin



(Saavedra et al.,1976), the parafollicular cells of the thyroid gland, and the pancreas (Koevary et al.,1983) of the rat and golden hamster (Lundvist et al., 1978). The pineal gland which is involved in the control of melatonin secretion, the end product of 5HT methylation, also contains very high concentrations of 5HT, both in the pinealocytes cytoplasm, and in the pineal sympathetic nerve endings. However, of major interest in this work, and to be discussed later, are the studies by Verhofstadt et al.,1983 and Holzwarth et al.,1983 showing localisation of 5HT stores in the chromaffin granules of the rat adrenal medulla.

1.3 BIOCHEMICAL PROPERTIES OF SEROTONIN

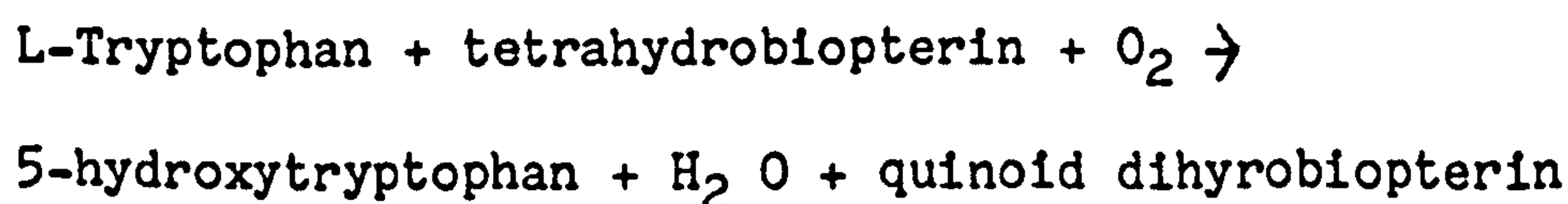
The systematic chemical name for serotonin is 5-hydroxy-3-(2-aminoethyl) indole and its chemical structure consists of a diffuse π electron cloud above and below the heteroaromatic plane which is pulled towards the aminoethyl group at position 3 making this the most basic region of the molecule . At pH 7.5 the molecule is completely protonated (cationic form) whilst at pH 10.5 approximately 30% each of the neutral form and the zwitterionic form exists (see Fig. 1.1). At alkaline pH, 5HT in solution is rapidly destroyed when heated and because of its instability as a base it is commercially sold as the creatinine sulphate salt. However at low pH e.g. pH 5.6, 5HT has good stability even at elevated temperatures. Serotonin exists as three other stable salts the picrate, hydrochloride and salicylate. The creatinine sulphate salt which

has a molecular weight of 405.4 ($C_{14} H_{23} N_5 O_7$), has a very high solubility in water which increases with temperature. In contrast it is only sparingly soluble in lipids. Three different dissociation constants have been determined related to the creatinine moiety ($pK_{a1} = 4.9$), the terminal amino group ($pK_{a2} = 10$), and the hydroxy group within the benzyl ring ($pK_{a3} = 11.1$).

1.4 BIOSYNTHESIS OF SEROTONIN

Approximately 2% of tryptophan is utilised in most animals by the gut, in the synthesis of serotonin. The gut is equipped with a transport carrier system in the enterchromaffin cells which actively transports the amino acid into the cytoplasm of the cell. Hydroxylation of both the D- and L-tryptophan (the naturally occurring amino acid), requires the enzyme tryptophan hydroxylase. This enzyme has been isolated in e.g. the venom producing glands of the toad (Udenfriend et al., 1953) but has been found in variable amounts in all tissues that contain 5HT except platelets, which store but do not synthesise serotonin.

The reaction catalysed by tryptophan hydroxylase (Fig.1.2) is:



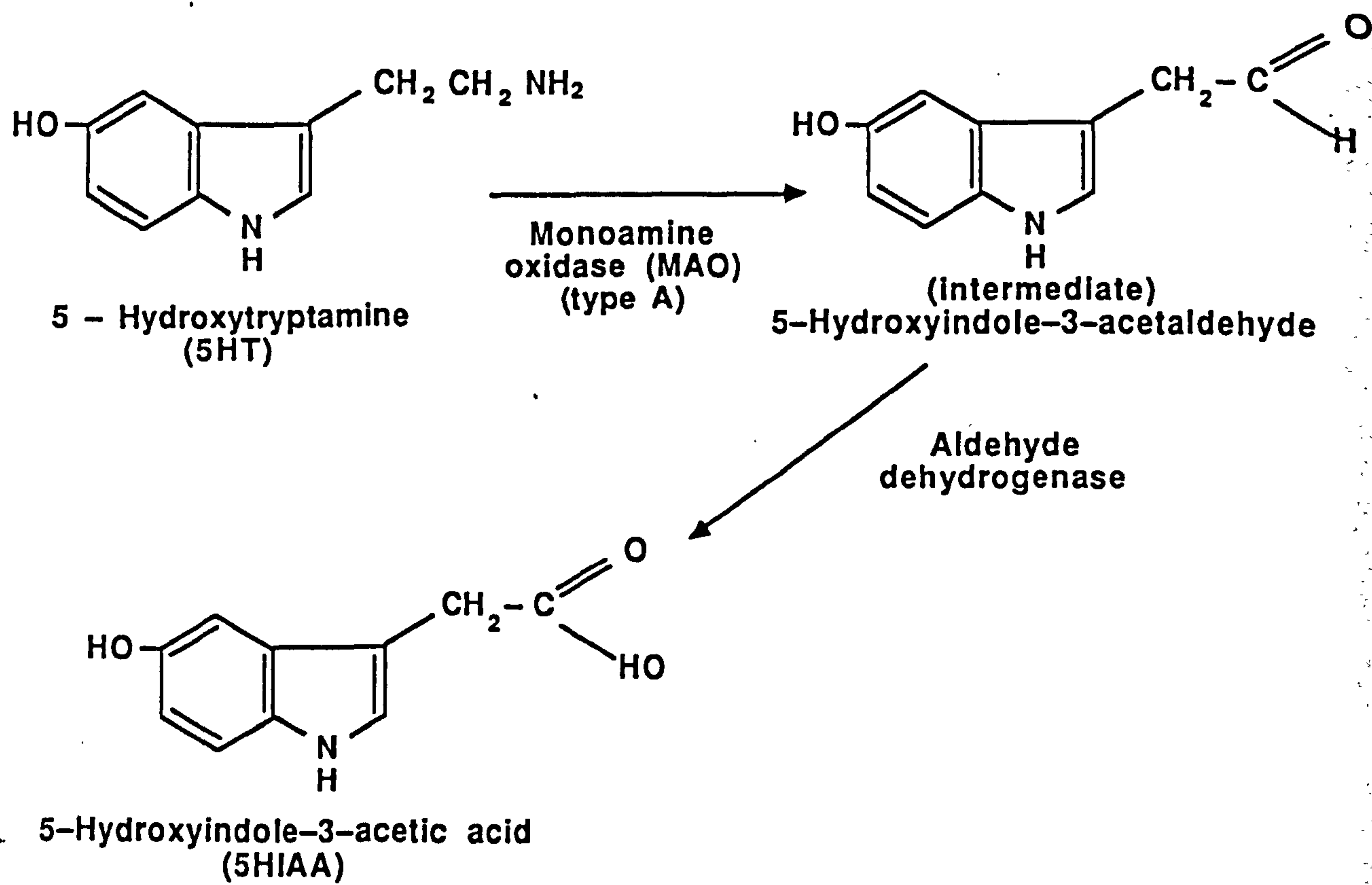
The Michaelis constant (K_m) of the enzyme is fairly high ($3 \times 10^{-5} M$) and under normal circumstances the concentration of tryptophan would be far below the K_m value so that in vivo the enzyme would be sub-saturated. However, at concentrations of $2 \times 10^{-4} M$ tryptophan the

enzyme is inhibited. Tryptophan hydroxylase requires Fe^{2+} as a cofactor and is easily inactivated by molecular O_2 so that sulphhydryl compounds such as dithioreitol which are potent reducing agents, are added to enzyme assays containing tryptophan hydroxylase.

Other important inhibitors of this enzyme include the amino acid p-chlorophenylalanine which is a competitive inhibitor of the enzyme in vitro, and in vivo is a potent long lasting inhibitor of 5HT synthesis. It has been extensively studied and used in depleting animals of central and peripheral stores of 5HT e.g Koe and Weissman 1966. Because the rate limiting step in 5HT biosynthesis is the hydroxylation of tryptophan, a diet poor in tryptophan will be reflected in lower stores of 5HT; the converse is true for high tryptophan consumption.

The next important step in 5HT biosynthesis (see Fig.1.2) requires the enzyme L-aromatic amino acid decarboxylase. This enzyme is capable of decarboxylating several amino acids including, phenylalanine, dihydroxyphenylalanine, tryptophan, tyrosine and specifically L-5-hydroxytryptophan. The enzyme is present in several animal tissues including the mammalian kidney and liver. The kinetics of the enzyme have however been determined from crude cat liver, kidney and adrenal extracts. The K_m for L-5-hydroxytryptophan decarboxylase is approximately $8 \times 10^{-6} \text{M}$ but the enzyme has a lower activity in the brain compared to the liver and the adrenals. It requires pyridoxal phosphate as coenzyme, which also inhibits the enzyme when present in excess. The decarboxylation of 5-hydroxytryptophan is not rate limiting and

Fig 1.3 Metabolism of serotonin



administration of 5-hydroxytryptophan to humans and rats for example, increases 5HT levels in several tissues including the gut and brain.

1.5 METABOLISM OF SEROTONIN

Serotonin is most commonly oxidised to 5-hydroxyindole-acetic acid (5HIAA). This requires oxidative-deamination and the reaction is attributed to the enzyme monoamine oxidase (Fig.1.3). This enzyme is ubiquitous and is found tightly bound to the outer surface of the mitochondrial membrane (Tipton et al 1976). It exists in two forms, monoamine oxidase A and B, each identifiable by the compounds that differentially affect their activity. Monoamine oxidase A is found both intra- and extra-neurally, whereas type B is solely extra-neural. Type A is specifically responsible for the oxidative deamination of 5HT and norepinephrine and type B deaminates a series of other amines including dopamine, and phenylethylamine. In the first step 5HT is deaminated to the intermediate 5-hydroxyindole-3-acetaldehyde by the enzyme monoamine oxidase (Fig.1.3), found in many tissues including the brain (Erwin et al., 1978). Like many other oxidizing enzymes which are involved in degradation processes it requires the cofactor NAD^+ . At this point in the pathway the aldehyde product can be shunted into what is a minor route of metabolism in mammals, to form 5-hydroxytryptophol. The compound is found in several peripheral tissues and is increased by the consumption of large amounts of alcohol due to a shunting from the oxidative pathway to the reducing pathway. More commonly however, 5-hydroxyindole -3-acetaldehyde is oxidised to 5HIAA by

aldehyde dehydrogenase. In the presence of the cofactor NAD^+ , 5HIAA is the major excretory product in mammals and is used as a marker in several clinical conditions briefly reviewed below.

The liver contains an important detoxifying enzyme, glucuronyltransferase, which is capable of conjugating glucuronic acid to 5HT as well as several other compounds including amino sugars and lipids. Serotonin-O-glucuronide is a major metabolite in the isolated rat liver perfusate and if monoamine oxidase is inhibited 5HT is readily converted to this product.

Serotonin can also be conjugated to a sulphate moiety and the enzyme responsible, serotonin-sulfotransferase, has been localised in liver, lung, kidney and brain tissue. When 5HT is sulphonated it loses biological activity but this may be only temporary since an enzyme capable of dissociating the sulphate moiety exists called arylsulphatase. However serotonin-O-sulphate accounts for only a small proportion of the total 5HT metabolites and although some has been detected in rat spinal cord and in the urine of normal individuals, its concentration does not increase markedly even when monoamine oxidase is blocked.

Serotonin in the pineal gland serves a special function of being the precursor to an important hormone called melatonin or 5-methoxy-N-acetylserotonin, which is responsible for blanching the skin of amphibians and suppressing gonadal activity in mammals. In this pathway, 5HT is converted first to N-acetylserotonin by the enzyme N-acetyltransferase also present in other regions of the brain. The enzyme requires acetyl coA to transfer a methyl group to 5HT. The

activity of this enzyme is under diurnal influence and is 15 times more active during the dark phase in parallel with an increase in melatonin synthesis and depletion of 5HT precursors.

The other major enzyme under diurnal influence and present in the pineal gland is hydroxy indole-O-methyl transferase. This enzyme requires S-adenosylmethionine for the transfer of a methyl group to position 5 on the 5HT molecule. Normally it uses N-acetylserotonin as substrate to form the end product, melatonin; however the enzyme can act on 5HT to form 5-methoxyserotonin and this reaction has been detected in the hypothalamus of the rat (Green et al 1973). N-methylation of 5HT can also occur, the enzyme requiring the same methyl donor as above. The enzyme exists in the rabbit brain and lung and the N-methylated 5HT products are psychotoxic e.g. the hallucinogen bufotenin (N,N-dimethylserotonin) can be formed in vitro by the action of brain N-methyltransferase enzyme in the presence of 5HT and S-adenosylmethionine, and may be of relevance in some mental diseases in which 5HT may play a major role e.g. schizophrenia.

1.6 MAIN STORAGE SITES FOR 5HT

1.6.1 Serotonin in the CNS

In mammals, 5HT is localised within nerve terminals of discrete brain regions, including the raphe nuclei of the brain stem (Osborne et al., 1982). The raphe nuclei are regarded as important sleep-inducing centres and selective destruction of this region in rat studies has led to a parallel depletion in 5HT content in the brain with

concomittant wakefulness, the loss of 5HT being proportional to the number of hours sleep loss. Also of importance, are the rostral nuclei which are important innervators of the hypothalamus. In this instance 5HT may be an important regulator of temperature control e.g.in the rat, injections of 5HT into this area results in a reduction in body temperature, but similar protocols in cats, dogs and monkeys produced an increase in body temperature, thus the effect is species dependent. Another area of the brain containing very high concentrations of 5HT is the hypothalamus . The hypothalamus is involved in the control of pituitary hormone secretions by the release of regulatory factors into the portal blood system. Serotonin is thought to act as a neurotransmitter in controlling the release of some of these factors including CRF (see section on PCPA). In clinical conditions such as Cushings' syndrome where plasma renin is low, treatment of patients with the 5HT antagonist, cyproheptadine, ameliorated the symptoms by reducing ACTH levels (Krieger et al 1977). Circadian surges of ACTH and corticosteroids have been dampened by depletion of brain 5HT levels with PCPA (Krieger et al.,1970,1977., Vernikos-Danellis et al., 1973). In man 5HT itself has been shown (Sauerbier (1976) see Smythe 1979), to follow a circadian pattern in whole blood, low levels of approximately 100pmol/ml maintained from 6pm-6am and reaching a peak at approximately 2pm of 540 pmol/ml. Corticosteroids themselves have been shown to directly influence 5HT synthesising enzymes e.g.tryptophan hydroxylase at the level of the hypothalamus both in vivo and in vitro (Azmitra et al., 1974), and thus by

modulating their kinetics, alter the secretion of CRF and consequently ACTH. Serotonin at the hypothalamic level may also be responsible for affecting compensatory adrenal hypertrophy in the contralateral adrenal of an adrenalectomized rat. Removal of the left adrenal prior to PCPA treatment in the rat, enhanced right adrenal hypertrophy and this was thought to be related to a possible effect of 5HT on ACTH secretion which affects adrenal growth, and a more direct effect of 5HT from the hypothalamus (Vernikos-Danellis et al 1973). Thus it would appear that 5HT in the CNS is involved in several regulatory processes.

1.6.2 Serotonin in the Gut

The alimentary tract of mammals contains vast stores of serotonin. Classically 5HT has been identified in the endocrine cells of the gut, the enterochromaffin cells, by immunohistochemical staining techniques, fluorescent staining and autoradiography. Stimulation of the release of 5HT is caused by acetylcholine, itself released by transluminal pressure, and a lowering of pH. It has been suggested that 5HT may be a local paracrine gut hormone involved in intestinal secretions and peristalsis of the intestine. Like most tissues in which 5HT is stored, the cells from the intestine in rats have uptake mechanisms for 5HT and are also capable of synthesising it from l-tryptophan and this capacity is markedly increased in carcinoid patients. There is a rapid turnover of 5HT in the gut with a half-life of 10-17 hours compared to 33-48 hours in platelets, and this is reflected in large excretions of the 5HT metabolite 5HIAA -up to 9mg daily in human urine. The other main storehouse of 5HT in the gut

has recently been designated to the intrinsic network of neurons running through the gut musculature with an estimated 5HT content of 100 ng/g. Stimulation of these neurons by for example, release of 5HT from the enterochromaffin cells on the mucosal side of the gut which induces peristalsis by depolarisation of primary afferents, initiates neuronal release of 5HT on the serosal side of the gut lining. The 5HT released on the serosal side causes depolarisation of enteric ganglia by a slow excitatory post synaptic potential (epsp) and thus inhibits peristalsis.

1.6.3 Serotonin in the Blood

Platelets form the major storehouse of 5HT in the blood where it is stored in dense granules in close association with ATP and Ca^{2+} . It has been estimated that during release reactions of platelets e.g. coagulation, platelets release approximately 0.21-5.95nmol of 5HT per 10^9 platelets at the local sites of injury.

There are different indices for the measurement of 5HT in blood.

Humphrey et al., 1954 has measured 5HT in the dog, at levels of $0.4\mu\text{g}/10^9$ platelets and in whole blood $0.35\mu\text{g}/\text{ml}$. More recently 5HT has been measured by radioimmunoassay (RIA) (Kellum and Jaffe 1976) with values of $2.2\text{nmol}/\text{mg}$ platelet protein in the rat.

Approximately 90% of 5HT is stored as an inactive platelet bound form in normal conditions in humans, with adult levels measured by RIA at $341 \pm 27\text{ng}/10^9$ platelets (Kellum and Jaffe 1976); serum levels have been estimated at $70 \pm 20\text{ng}/\text{ml}$ (Snow et al., 1955) and $210 \pm 53\text{ng}/\text{ml}$ in another study (Davis 1959).

There have been suggestions that 5HT levels may alter with age since studies done by Erspammer in 1954 (see Smythe 1979) indicated higher levels in adults (mean 680pmol/ml; range 410-1140 pmol/ml) compared to elderly patients (mean 500 pmol/ml; range 170-850 pmol/ml serum). Plasma-free levels or platelet poor plasma (PPP) levels have been estimated by Crawford et al., 1963 with a mean free level of 13ng/ml (0-165pmol/ml) and similar levels have been reported by other groups. However the results are variable, and since platelets are fragile, any slight damage to them will cause release of 5HT into the plasma. Thus, on this basis it is not clear whether the values represent free 5HT, or an artefact due to platelet release. Furthermore, it has been suggested that when blood clots only part of the 5HT in platelets is released and therefore the values quoted for serum may not be constant. However, Davis et al., 1959 reported constant recovery of 5HT if blood was able to clot in the presence of ascorbic acid.

Variations in 5HT levels are prevalent immediately after a meal and increase up to twice the normal fasting levels (Kellum and Jaffe 1976) one hour after food rising from a resting value of 1130 ± 210 pmol/ml to 2060 ± 97 pmol/ml.

There have also been studies on a limited number of individuals which suggest that there may be a circadian rhythmicity in 5HT, the levels being higher in the early evening and early morning, and lower in midmorning and afternoon (Genefke et al., 1968) in parallel to ACTH rhythms.

Serotonin reaches platelets via the gut. In the intestine 5HT

ingested or synthesised from the amino acid tryptophan is secreted into the gut lumen and transported in the portal vein system to the liver where it is inactivated by monoamine oxidase A. The gradient of 5HT from the portal vein to the hepatic vein leaving the liver is 30% but any 5HT that may escape deactivation is rapidly transferred to the pulmonary circulation to be degraded in the lung parenchymal tissue. The ability of the lung to remove 5HT was first observed 60 years ago. It has now become apparent that over 90% of 5HT is deactivated in the lung by monoamine oxidase A. This is one of the major non-aerobic functions of the lung. The lung has a large surface area /volume ratio and a large endothelial cell capacity which inactivates serotonin. Besides endothelial cells, trapped platelets, mast cells (in rats and mice), and adrenergic nerve endings may also contribute to a lesser extent to the storage and deactivation of 5HT. In the case of platelets, 5HT is taken up over longer periods of time than the 4-10seconds transit time through the lungs in which inactivation occurs.

After passage through the lungs 95% of the 5HT passing through the lung, is removed and the remaining 5% passes to the left atrium to enter the general circulation.

The measurement of 5HT in tissues is more a consequence of overall storage versus release or synthesis versus metabolism so that in order to draw up a true identity of its physiological function one would require dynamic studies of turnover (see Chapters 8 and 11).

1.7 PATHOPHYSIOLOGY OF SEROTONIN

1.7.1 Carcinoid Syndrome

As outlined above 5HT is stored in the enterochromaffin cells of the gut. The concentration varies along the regions of the gut and it has been reported that substance P may be a major co-storage amine in these cells. It has been known for a long time that in the Carcinoid Syndrome there is tumorous growth of these cells resulting in overproduction of 5HT; indeed it has been shown that over 60% of ingested tryptophan may be taken up by these cells for 5HT biosynthesis. In this syndrome one of the classical symptoms is watery diarrhoea as a result of excessive peristalsis and increase transit time reducing absorption. Blood levels of 5HT may also be elevated in this condition up to 5-10 times whilst the urinary excretion product 5HIAA can be elevated by as much to 60 times the normal (Sjoerdsma, Weissbach and Udenfriend 1956 - see Smythe 1979). For this reason urinary measurements of 5HIAA are made in the differential diagnosis of this intestinal disease.

Other intestinal diseases may also manifest abnormal handling of 5HT with elevated levels in the blood and produce clinical symptoms related to 5HT of flushing, diarrhoea and wheezing.

1.7.2 Hypertension

In essential hypertensive patients platelet uptake of 5HT is reduced either due to abnormal uptake systems or a more rapid turnover of platelets due to increased aggregability (reviewed by Vanhoutte 1982). In clinical conditions, such as pulmonary embolism or in

spontaneously hypertensive rats (SHR) where there may be pulmonary oedema, there is decreased capacity to inactivate 5HT and therefore the blood 5HT levels may increase. Guicheney and coworkers (Guicheney et al., 1985) in studies using normal rats and SHR demonstrated an increase in blood platelet concentration in the SHR with no change in 5HT content per platelet number. This was associated with an increase in aggregability of the platelets. Vanhoutte's review indicated that this could create a positive feedback effect of 5HT in that more release from platelets of hypertensive animals, would be further augmented by 5HT's capacity to increase platelet aggregation. This increase in platelet turnover was associated with an increase in free tryptophan levels. An increase in platelet turnover means that stored 5HT is released more rapidly from the platelets and so there is a net requirement for 5HT synthesis. Hence the essential amino acid tryptophan, is made freely available for uptake into the synthesising machinery of the enterochromaffin cells. Studies in Man by Baudouin-Legros et al., 1985, showed that the 5HT content in platelets from hypertensive patients was lower than in normal subjects. Kamal et al 1984, has also reported a diminished uptake transport system in platelets from hypertensive subjects. Roth et al 1980, suggested by his studies on perfused normal and hypertensive rat lungs, that there is a reduction in clearance of 5HT in the endothelial cells because of slower uptake, rather than a change in the content of the monoamine oxidase responsible for oxidative deamination of 5HT in these cells.

The overall effect in aggravating or contributing to the hypertensive

state of reduced uptake and metabolism of 5HT in animals, has been summarised by Vanhoutte et al 1982 as an overall increase in blood vessel wall sensitivity to 5HT and a resultant increase in blood pressure.

More recently a study carried out by Osunkwo et al., 1986, measuring 5HT levels in the genesis of hypertension in rats, indicated that an increase in 5HT may be more important in deoxycorticosterone acetate (DOCA)-induced hypertension at its onset, but is increased in several hypertensive states including spontaneous hypertension, renal hypertension and DOCA-induced hypertension during the later phases. However, like previous studies it has not been determined whether changes in 5HT induce the hypertension or whether the change in 5HT handling is a consequence of the hypertensive state.

1.7.3 Central Nervous System Disorders

Primary aldosteronism is characterised by high plasma levels of aldosterone but suppressed levels of renin. It has often been broadly classified with adrenal hyperplasia but in this case there is adrenal hypertrophy not seen in primary aldosteronism. There is evidence that 5HT may be involved either peripherally or at the level of the central nervous system in stimulating excessive aldosterone release from the adrenal. Gross et al., 1981, showed that in humans with primary aldosteronism treatment with the 5HT antagonist, cyproheptadine, by i.v. administration, suppressed aldosterone secretion and further, the effects could be accentuated by the pre-infusion of a peripheral inhibitor of 5HT synthesis carboxydopa.

This suggested to Gross that the effects of 5HT in primary aldosteronism were centrally mediated. Mantero et al., 1985 using a more specific 5HT antagonist, ketanserin, given i.v. to patients with idiopathic hyperaldosteronism and adrenal adenoma showed that aldosterone levels in the adenoma patients were decreased significantly in 2 out of 4 patients (not seen in adenoma patients by Gross) whereas in 4 out of 6 patients with idiopathic aldosteronism there was only a slight decrease in aldosterone levels. However the percentage change from individual basal values showed a slight but significant drop in aldosterone levels. This study did not distinguish between the two disease states or differentiate the site of 5HT's possible action.

Chapter 2

THE ADRENAL CORTEX: CONTROL OF GROWTH AND NEURAL INNERVATION

2.1 BACKGROUND

The adrenal glands of the eutherian mammal are paired structures lying in the abdominal cavity close to the anterior pole of each kidney . They are surrounded by both brown and white fat and vary in shape from almost spherical, in foetal life and in the adult species of the rat, mouse, and guinea pig, to wedge- shaped flattened structures in humans and cattle. Although the size of the gland may vary with species, the thickness of the cortex remains constant and this is thought to be due to the arrangement of the blood supply which is similar throughout the eutherians, in that there is only a single vein leaving each gland which results from the centripetal blood flow within the gland . Accordingly, any increase in cortical width would cause the venous end of the capillary bed to be too far from the arterial supply.

The mammalian adrenal gland consists of two components, adrenocortical and chromaffin tissue. In embryonical development, adrenocortical cells arise from the mesoderm, in particular from the columnar epithelial cells that line the coelom. Chromaffin cells have a separate origin, arising from the paraganglion cells of the neural crest complex. The concentric arrangement of adrenal cortex surrounding the medulla is found only in eutherian mammals among the vertebrate class. There appear to be important interactions between the medulla and the cortex. It is not known what attracts the medullary cells to the cortex but there is little doubt that the glucocorticoids play an important role in medullary cell maturation e.g. sympathetic tissue from animals of various ages cultured in

medium with glucocorticoids, preferentially differentiate into adrenal medullary cells (Coupland et al, 1965). Further, neonatal rats possess clusters of chromaffin cells that surround the aorta (the organ of Zuckerkand) which atrophy after birth but can be maintained with administered glucocorticoids during the neonatal stage. Other studies (Wurtmann et al, 1966) indirectly demonstrated the importance of glucocorticoid control of catecholamine synthesis when it was discovered that the enzyme activity of phenylethanolamine N-methyltransferase (PNMT), was decreased in hypophysectomised rats and also in rats where steroid production was inhibited. They concluded that the effects of ACTH release on the secretion of glucocorticoids were important in the activity and biosynthesis of epinephrine from norepinephrine. Conversely, medullary tissue and its hormone secretions may augment or maintain the cortical cells and their secretions, for example the medulla has been shown to complete the synthesis of cortisol when medullary tissue is incubated with C-21 and other steroid precursors, (Carballeira and Venning 1964). In contrast, epinephrine has been shown to inhibit the synthesis of cortisol when added to adrenocortical mitochondria by reducing 11-beta hydroxylase activity (Sweat and Bryson 1965.) The addition of ascorbic acid prevented this effect, thus indicating that an oxidizing agent was responsible for the inhibition of the enzyme. This could conceivably be the metabolic end product of epinephrine, adrenochrome. This fine control of steroid synthesis by catecholamines, may help to regulate the outflow of glucocorticoids and their precursors especially during stress. In relation to this,

it has been discovered that medullectomised animals, maintain basal levels of glucocorticoid output but have a reduced steroid response to immobilisation stress (Torda and Kvetnansky 1983); further to this it has been observed that immobilisation stress in rats depletes catecholamine levels in the adrenal medulla (Kuriyama et al 1984). However in vivo perfusions of epinephrine in intact animals have varied from having no effect on basal glucocorticoid secretions in man and isolated perfused dog adrenals, to reducing the corticoid response to ACTH (Sandberg et al., 1953., Cushman et al., 1966) whilst Vogt et al., 1944 showed an increase in steroid output of isolated dog adrenals which was independent of the pituitary but dependent on the presence of medullary tissue. A recent explanation has been postulated by Pratt and coworkers (Pratt et al 1985) which explains the lack of effect in isolated fresh adrenal cells and in vivo perfusions of catecholamines, as due to the high local concentrations of the catecholamines within the cortex leading to desensitisation. Cultured zona glomerulosa cells from bovine and rat are stimulated by norepinephrine and epinephrine to secrete aldosterone, (Pratt et al., 1985., De Lean et al., 1984b.,) and have low endogenous levels of catecholamines, whereas freshly prepared rat zona glomerulosa cells, appear to have higher endogenous levels and do not respond to catecholamines (Sequeira et al., 1985), thus, a desensitisation effect could explain some of the variable results.

2.2 SYSTEMIC EFFECTS OF ADRENAL CATECHOLAMINES ON HYPOTHALAMIC-PITUITARY-ADRENAL FUNCTION

Catecholamines exert effects both at the level of the pituitary via the hypothalamic - hypophyseal portal system and also via the systemic circulation from the adrenal medullary release. It is well recognised that epinephrine infusions have a significant effect on pituitary function and may reach levels as high as $10^{-8}M$ during chronic stress. Indeed, infusions of epinephrine have been used in clinical studies in discerning pituitary-adrenal axis function (Sandberg et al., 1953). Direct in vitro studies using cultured pituitary cells have shown that epinephrine can stimulate the release of ACTH via alpha receptors (Giguere et al., 1982) . At the higher level of control, the release of corticotrophin releasing factor (CRF) from the hypothalamus, is stimulated by epinephrine and inhibited by norepinephrine (Jones and Hillhouse 1977). However the overall effects are variable and complex with reports of falls in ACTH levels with epinephrine infusions (Wilcox et al., 1975) to an early fall and later rise in ACTH secretions reported by Few et al., 1980.

2.3 BLOOD SUPPLY

The rat and guinea pig give typical examples of the blood supply arrangement to the adrenal gland. In general the arterial blood is derived from several vessels. In the rat, the anterior part of each gland is supplied by an artery arising from the aorta which divides into two branches, and after further sub-division ramifies across the

surface of the gland without apparent anastomoses. This compares to the cat which has numerous small arteries arising from the aorta. In man the major portion of the blood supply to the adrenal is derived from the inferior adrenal arteries arising from the renal artery.

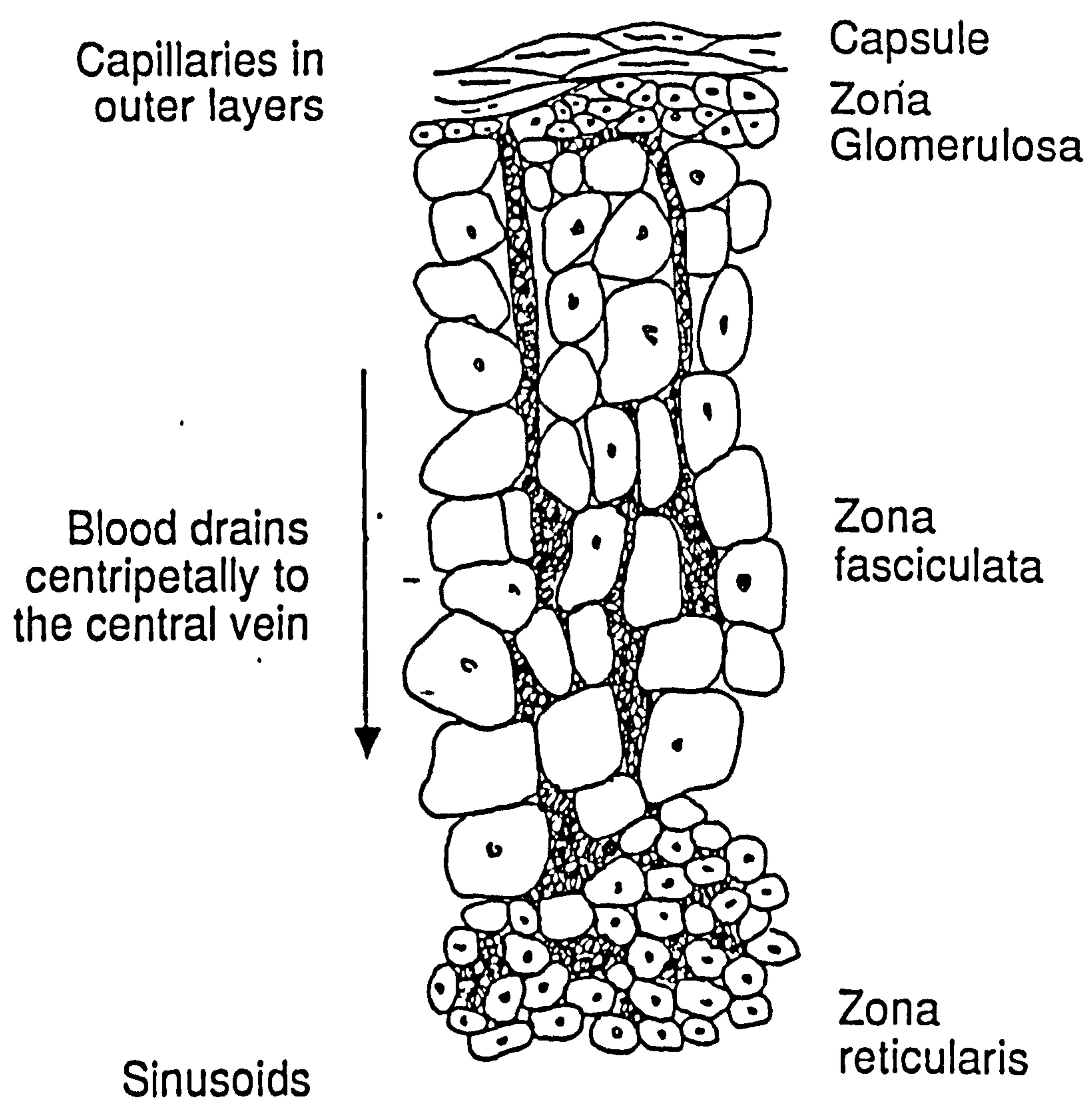
2.4 VASCULAR PATTERN WITHIN THE ADRENAL

In 1900 Flint described the pathway of the blood vessels within the gland by use of the injection of dye to follow its pathway (see Idelman 1978). The adrenal arteries form a network across the capsule to supply the whole gland and may either :-

- (1) be confined to the capsular region and terminate in an irregular capillary network which is gathered into anastomosed venous channels;
- (2) penetrate into the cortex and immediately break up into a vast capillary network confined to this region - arteriae corticis;
- (3) penetrate the cortex without branching and then branch out into a capillary network within the medullary region- arteria medullae.

The cortical vessels that penetrate (2) initially break up into straight capillaries that embrace the zona glomerulosa cells forming anastomoses of the 'arcade' type. Within the zona fasciculata the capillaries become straighter and follow the connective tissue septa that runs radially within the gland towards the inner cortical region, the zona reticularis. Here the vessels widen into anastomising blood spaces and sinuses that drain into the central vein. The capillaries of the cortex are of the sinusoid type, which includes both endothelial cells and histiocytes. These vessels due to their composition, have the capacity to store exogenous matter

Fig 2.1
Cross sectional view of the structure of the adrenal cortex



Taken from
Endocrinology (1986) by CRW Edwards

including india ink, protein, trypan blue and silver nitrate (see discussion in Chapter 11).

2.5 MICROANATOMY OF THE ADRENAL CORTEX

Arnold in 1866 (Idelman, 1978) first described the histology of the adrenal cortex with its three concentric zones, the zona glomerulosa (glomus-ball), zona fasciculata (fascis-bundle) and zona reticularis (rete- net). The organisation of these zones is due to the growth and organisation of the internal blood vessels (see below). Under the light microscope the zona glomerulosa may be well defined in some species such as dog, sheep and horse and less defined in monkey, rat, hamster and mouse. The zona reticularis may not be prominent in some species such as the mouse, and may be marked in others e.g.pig and guinea pig. The volume of the gland varies with the species and the physiological state of the animal. In man the zona glomerulosa occupies 15% of the total gland compared to 38% in the rat and 20% in the ox. Similarly, the zona fasciculata occupies 78% in man ,54% in the rat and 51% in ox. The rat is exceptional in having a zona intermedia region occupying 3% of the gland.

2.6 MORPHOLOGICAL CHARACTERISTICS OF EACH ZONE

The zona glomerulosa lies just inside the capsule and consists of cells arranged in ball-like clusters 20-30 cells per cluster with a zonal width of 4-6 layers (Fig. 2.1). The cells approximate 13 μ m diameter with a high nuclear/cytoplasmic ratio. The nuclei vary in shape and contain one or two nucleoli. The cytoplasm contains a

variable amount of lipid, being particularly high in the dog, rat and hedgehog (as reviewed by Idelman, S. 1978).

The zona fasciculata cells lie below the zona glomerulosa layer and are arranged in columns radiating towards the zona reticularis and medulla. They lie juxtaposed with the connective tissue septa and intermittently interrupted by the sinuses passing through the cortex. The cells are larger than the zona glomerulosa cells with a diameter of approximately 21 μm and have round nuclei with one or two nucleoli and although often being called 'clear cells' because of their vacuolated appearance in tissue sectioning, they do in fact contain a large amount of lipid within the cytoplasm compared to glomerulosa cells. Frequently the lipid droplets are more numerous and larger in the outer layers than in the inner fasciculata layers. Interdigitating with the fasciculata zone lies the zona reticularis which is smaller in width. The cells are arranged in cords that anastomise and surround the large blood sinuses. The cells are small and lipid content is sparse. There has been some speculation on the apparent dichroism of zona reticularis cells since as early as 1886 Dostoiewsky (see Idelman 1978) had noted that there were two types of cells, light or clear cells, and dark or compact cells. A modified theory expounds that the clear cells in this region and the zona fasciculata may provide a precursor steroid store, and compact cells provide the immediate steroid requirements. Therefore after ACTH stimulation the clear cells transform to compact cells to give increased output of steroid (Symington 1962). Studies with prolonged ACTH administration indicated marked hypertrophy of the

zona reticularis, and a dense nerve plexus was evident in studies carried out by Mikhail (1961, 1965) and in several mammals including the rat and mouse (Migally 1979). This indirectly suggests that nerve supplies in this region, thought to affect adrenal growth, may be regulatory in the zona reticularis.

2.7 CONTROL OF GROWTH AND MASS OF THE ADRENAL CORTEX

The rate of secretion or production of a given hormone is the product of the activities of the enzymes involved in its synthesis and the mass of the adrenal cortex involved in this process. Thus the mass of the cortex is an important means of controlling the steroid output in the different zones.

In the case of aldosterone, the mass of the zona glomerulosa is solely responsible for the secreted amount in all mammals since aldosterone synthesising enzymes are specifically located in this region. However, in the case of corticosterone and cortisol secretion, the zona fasciculata, zona reticularis, and in the case of corticosterone, the zona glomerulosa to a lesser extent, are all involved in the production of these steroids. Hence the mass of the adrenal cortex as a whole, becomes the determining factor. In some species, androgen production becomes an important secretory product of the inner zones including the zona fasciculata and the zona reticularis e.g. in human foetal and adult tissue these regions actively secrete androgens and there is a concomittant increase in adrenal mass, reflective of the increased mass of the inner zones.

2.7.1 Control of the Mass of the Cortex

Although as discussed above the mass of the zones are important determinants in the secretion of specific steroids, this may not be a direct regulatory mechanism but rather a slower feedback control mechanism, secondary to a change in zonal width function. The zonal width may be argued as being controlled by dynamic changes in the functional and morphological characteristics of the cells constituting the zones, as has been discussed by Hornsby and coworkers (Hornsby and Crivello 1983., Hornsby P.J., O'Hare M. J., and Neville A. M. 1974), and will be discussed further under the heading 'Functional Zonation'.

2.7.2 The Origin of the Growth of the Adrenal Cortex

The basic pattern of growth is similar in all mammals and only differs in detail such as occurs in the X zone in the female mouse or the human foetal zone that later convolutes. In general the adrenal gland assumes a spherical shape with layers of cells clustered together so that realistically, cell division is most likely to be restricted to the outer regions of the zona glomerulosa. In fact two theories have been postulated to account for cell division within the cortex, each of which may not be totally exclusive. Originally Gottschau 1883 (Idelman, 1978) postulated the 'cell migration' or 'escalation' theory in which cells divide in the outer zones and migrate inwards and are progressively transformed into the cells characteristic of the different zones. Since then several groups have shown that cell division does occur more markedly in the outer

regions. Indeed, pulse chase experiments in young rats stressed with carbon tetrachloride which stimulates adrenal growth, and later injected with [^3H]-thymidine, revealed that cell division in the outer cortical regions was more marked in 2 and 3 week old rats compared to one and four weeks (Jones (1967) cited in Idelman 1978), and further, tracer experiments in 6 day to 10 week old rats showed that [^3H]-thymidine could be located at different regions further into the cortex with time, reaching the zona reticularis by the end of two months (Ford and Young 1963). Others have also found that in both embryonic and postnatal growth, most cell division appears to occur in the outer zona glomerulosa and outer zona fasciculata (Wright 1971, 1973). The situation is similar in the mature cortex with a predominance of division in the outer zone. However the rate of cell division is less in the mature gland, yet enough to balance the rate of cell death. Wright in his studies in the rat, (Wright 1971, 1973), found that the rate of cell division in the zona glomerulosa was higher than that required for its own maintenance, whereas the rate of cell division was too low in the zona reticularis for maintenance, thus reinforcing the idea that the outer cortex is of primary importance for whole cortical growth. The zona reticularis has often been called the 'senescent' zone and deposits of large amounts of age pigments (lipofuscin) are found here. Lipofuscin is thought to be a product of the lipid peroxidations that occur in the outer zones as steroids interact with zonal enzymes (see later). Apoptotic bodies (dead cells) are also found in this zone, so it would seem a reasonable proposition that zonal maintenance is

achieved by migration of dividing cells in the outer zone. There are however, reports of zona reticularis cells actively dividing, for example [³H]-thymidine studies by Riter and Hoffman in 1967 (see Idleman 1978), showed that cell division occurs in this region; further, when adrenocortical tissue fragments were transferred to another region of the body, regeneration and functional and morphological zonation ensued. In bovine adrenocortical cells in primary culture, all cells entered DNA synthesis and were proliferating (Hornsby and Aldern 1984). All zones therefore appear to be capable of regeneration when required even though the inner regions appear to be quiescent or less active, possibly due to their location relative to the vasculature. The zona glomerulosa layers being closer to the arterial blood supply may have more access to oxygen and nutrients and circulating growth factors than the inner zones and because of this, are in the main, the most mitotically active. This latter idea of separate integrity of the zones and their maintenance was crystallised by Deane and Greep in 1946 in the 'Zonation Theory' and in essence they postulated that each zone was a functional and morphologically separate zone. This theory however is too limiting for adequate interpretation of the experimental findings to date and the concept which has emerged, considers that cells dividing in the outer zona glomerulosa zone are pushed inwards towards the medullary region, and in the process are undergoing a functional transition from mineralcorticoid secreting, to glucocorticoid, to androgen secreting and finally, to cell death in the inner zone. This idea is interwoven with the role played by the

vasculature and its blood-borne substance/s in zonal integrity, discussed in later sections.

2.8 STIMULATION OF GROWTH OF ADRENAL MASS

There are two parameters that can be included under the heading growth:-

- (1) adrenal cell size;
- (2) adrenal cell number.

2.8.1 ACTH

Adrenocorticotropin (ACTH) is the major regulator of adrenocortical cell growth in vivo. A classic example of this is in Cushing's syndrome in which an excess of ACTH secretion is associated with adrenal hyperplasia producing an increase in cell size rather than cell number or DNA synthesis. Conversely blocking of ACTH secretion by administering glucocorticoids or removal of the pituitary causes adrenal atrophy (Wright, Appleton and Morley 1974). In cell culture experiments ACTH alone inhibits cell proliferation, but in combination with a directly acting mitogen, cellular hypertrophy ensues (Gill et al, 1982) hence, in vivo the effects of high levels of ACTH would appear to be due to the indirect effects of ACTH as well as a directly acting mitogen possibly also derived from the pituitary or the hypothalamus (Holzwarth et al, 1980., Lowry et al, 1983)(see also other factors below). The sole effects of ACTH can however be seen in vivo when synthetic or natural ACTH is administered and there is a resultant increase in cell growth within 24 hours due to increase in cell size. In anencephalic fetuses where the pituitary

is absent, adrenal growth is poor but is restored by ACTH administration (Parker et al.,1983). Whether the effects are solely a result of ACTH's indirect mitogenic effects or possibly due to contaminating ACTH fragments such as pro-gamma MSH (Lowry et al.,1983) is not known.

2.8.2 Mitogenic Peptides

A family of polypeptides exist that are directly mitogenic to cultured adrenocortical cells. Fibroblast growth factors (FGFs) are the most potent and are found in both the brain and pituitary gland. They exert mitogenic effects on mesodermal tissue including the gut but their physiological role and the circumstances in which they may be stimulated are not known. Epidermal growth factor (EGF) also has mitogenic effects in cultured human foetal adrenocortical cells. It is secreted by the sub-mandibular gland and is also thought to influence medullary secretions. Binding sites in vitro in foetal rat tissue and also in vivo in adult rats have been identified (Crickard et al.,1981., Chabot et al.,1986) but the mitogenic and steroidogenic effects are species specific since there is no effect in bovine cells whereas steroid synthesis and secretion occurs for example, in sheep adrenals.

2.8.3 Pituitary Factors

Early work on crude pituitary fractions revealed that as ACTH concentrations were increased, so the mitogenic properties of the extract decreased. However, it has been demonstrated that growth factor alone (cited in Vinson's paper 1981) and in combination with

ACTH are mitogenic both in normal sodium balance and in sodium depleted rats (Palmore et al., 1970).

Hyperprolactinaemia also causes adrenocortical hypertrophy and indeed prolactin receptors have been found in the adrenal cortex. However in cultured adrenal cells both growth hormone and prolactin fail to stimulate mitogenesis (Gill et al., 1982).

It has also been shown that pituitary factors other than ACTH stimulate mitogenesis, for example, pituitary tumour cells and their secretory products have been shown to stimulate growth in Y-1 adrenocortical tumor line cells (Kudlow et al., 1983) and recent experiments have suggested that alpha MSH may also stimulate growth (Whitehouse et al., 1982). In cultured rat zona glomerulosa cells, Payet and coworkers (Payet et al., 1984), demonstrated a specific growth promoting effect of vasopressin, a hormone of the posterior pituitary gland.

2.8.4 Angiotensin II (AII)

It was shown by Mark et al., 1963 that chronic administration of angiotensin II in rats increased adrenocortical mass and sodium deprivation also increases the mitotic index of adrenals. Although angiotensin II receptors are in highest concentration in the zona glomerulosa, there is an increase in mitotic activity both in the zona glomerulosa and the zona fasciculata. The effects of AII may involve an interaction with ACTH since AII at the level of the pituitary may affect ACTH secretion.

2.8.5 Insulin and Insulin-like Growth Factors

Early work using bovine adrenocortical cells indicated the mitogenic effects of insulin, somatomedins, multiplication- stimulating activity (MSA), and insulin-like growth factors. Insulin type receptors have since been identified in vivo in rat adrenal cortex, (see cited reference in Hornsby, P. (1985) Chapter 1, Adrenal Cortex, Anderson and Winter 1985), although the specific effects in vivo have not been demonstrated. However, in vitro insulin and insulin -like factors appear to synergise with AII or FGF in stimulating mitogenesis (see Chapter 1, P.Hornsby, Adrenal Cortex).

2.8.6 Neural Involvement in Growth Regulation

As early as 1951, Kiss had suggested that neural innervation may in part, be responsible for adrenal growth after bilateral adrenalectomy, since they demonstrated an increase in size of cell bodies in the hypothalamic ventromedial nucleus, at the side opposite to adrenalectomy. Labelled [³H]-leucine uptake has also been shown to increase on the enlarged nuclei side. That stretch receptors may be involved in relaying afferent signals to the hypothalamus, was also suggested by Niijima (1968) and Dallman (Dallman et al., 1977). However, Holzwarth (1980) demonstrated that adrenal regeneration caused by unilateral enucleation procedures, i.e. removal of the inner cortex and medulla, resulted in compensatory adrenal growth which was independent of hypothalamic connections, since the observations were made in rats which had hypothalamic lesions; Thus, a dichotomy of both neural and humoral

2

involvement (possibly derived from the anterior hypothalamus) in the control of growth in unilateral adrenalectomy and adrenal enucleation respectively, has been proposed by Holzwarth and coworkers (1984).

2.8.7 Vascular Control of Growth

The vasculature has also been postulated by Hornsby (Hornsby, P.J.1985, chapter 1 in Adrenal Cortex, Anderson and Winter, eds) to indirectly control the size of the adrenal . It is already known that ACTH stimulates increased blood flow to the adrenal gland (Payet et al., 1980) by virtue of local prostaglandin release since indomethacin blocks the hyperaemia. This increase in blood flow may augment an increase in growth factors and nutrients that will increase adrenocortical growth. An acute increase in blood flow and hence volume of the gland could also trigger neural reflexes in the baroreceptors in the cortex and potentate growth via the neural-hypothalamic reflex in the contralateral gland and since both glands would receive an increased blood supply due to ACTH, both would increase in size. The chronic effects of an increase blood flow may involve an increase in capillary proliferation in the cortical tissue, stimulated by prostaglandins , thus increasing the flow of nutrients and factors such as FGF and EGF. Capillary proliferation may also produce an increase in secretion by endothelial cells of extracellular matrix, in particular fibronectin, which has potent growth stimulating properties and it has already been shown in cultured bovine and human foetal adrenocortical cells that coating of the substratum with extracellular matrix or fibronectin, increases

adrenocortical growth (Simonian, White and Gill 1982.,
Crickard et al.,1981).

2.9THE ZONATION OF ADRENOCORTICAL FUNCTION

Since the isolation and characterisation of aldosterone, the close association of the level of secretion of this steroid in altered sodium status and the width of the zona glomerulosa in relation to this has become apparent. Several groups have confirmed the specificity of the zona glomerulosa for aldosterone secretion in several species including man , rat and cattle (Tait et al., 1972,1975, 1980a, 1980b., Haning et al., 1970, Fraser et al., 1979, McKenna et al., 1978a, 1978b, 1979). In contrast, the inner zones secrete the glucocorticoids including cortisol in man, and corticosterone in the rat.

However, there are species differences in relation to some of the closely related 18 hydroxylated products thus in the rat, 18 hydroxycorticosterone (18OHB) and 18 hydroxydeoxycorticosterone (18OHDOC) are produced by the inner zones , in contrast to the ox. Deoxycorticosterone is a weak salt retaining hormone and was originally thought to be involved in salt and water balance in the 1940's and thus was thought to be located specifically in the zona glomerulosa but it has since been synthesised by addition of radioactive precursors such as progesterone and pregnenolone in the inner zones (Whitehouse and Vinson 1971, 1973). The specificity of steroid products was further investigated by the Tait's using isolated rat adrenal tissue perfused in a column system and comparing the

output of the inner zones to the capsular zones (Tait 1970). The secretory products 18OHB, 18OHDOC and B were products of the inner zone and subject to suppression in hypophysectomised rat adrenal cells. In contrast, the capsular cells secreted aldosterone and the other steroids in lower amounts but were unaffected by hypophysectomy thus indicating a dissociation of the zonal regulation and function. The reason for aldosterone production being confined to the capsular zone, relates to the presence of a cytochrome p450-dependent corticosterone methyl oxidase enzyme. The inner zones are capable of 18 hydroxylation of DOC and corticosterone (B) due to the 18 hydroxylating activity of the cytochrome p450 11-beta hydroxylase.

2.10 ZONATION HYPOTHESIS BASED ON VASCULATURE

Isolated cells from all zones of the adrenal gland lose their functional and morphological characteristics when placed in culture (Hornsby and Crivello 1983) and it has been postulated that this is a result of the removal of the vasculature. Greep and Deane had previously associated an important role to the vasculature in describing adrenocortical zonation. In support of this it has been recognised that the zona glomerulosa layer appears only on the arterial side of the vasculature e.g. in the human adrenal a cuff of cortex surrounding the central vein penetrates the medulla and a capillary plexus supplying the cortical cuff has an associated zona glomerulosa on the arterial side of the capillary bed. In adrenocortical tissue fragments that have regenerated, zona glomerulosa cells are found on the arterial side, whilst

adrenocortical tissue transplanted to the anterior eye chamber shows a zona glomerulosa layer on revascularisation, close to the arterial side. However in all these circumstances it is only assumed that functional secretions follow morphological characteristics. As indicated earlier, the cytochrome p450 CMO, is highly sensitive to steroid concentrations, and this may be an important criteria for zona glomerulosa appearance on the outer arterial side. It has therefore been postulated that it is the presence of a gradient of substance(s) carried in the blood supply, which alters the activity of the adrenocortical enzymes involved in steroidogenesis. In the case of cytochrome p450 CMO, activity was lost in cultured rat glomerulosa cells by the addition of cortisol and other steroids due to excess ACTH or cAMP stimulation, reverting the cells to zona fasciculata/reticularis characteristics and therefore not affecting the formation of corticosterone and 18-deoxycorticosterone; therefore cytochrome p450 11-beta activity was not affected compared to bovine adrenocortical cells in culture (Simonian et al., 1979). The way in which steroids may affect enzyme activity has been postulated by Hornsby and Crivello (1983) in which steroids may act as pseudo-substrates and cause the release of lipid peroxidases, which in the outer zones where antioxidants may be low, would cause inactivation of the enzymes required for aldosterone production. Further into the adrenal, other enzymes may be inactivated at higher concentrations of steroids e.g. 11-beta hydroxylase in the zona fasciculata of the rat, human and bovine cell cultures (Hornsby et al., 1982., Simonian et al., 1979) or 21-beta hydroxylase. This and other studies have

created a model in which the steroid concentrations themselves conceivably control the functional characteristics of the zones. Thus, in the inner zones a high steroid concentration of androgens e.g. in the human foetal adrenal, would stimulate ACTH secretion to increase glucocorticoid output, which would further augment androgen formation, creating a positive feedback loop where cAMP levels are high and preferentially stimulate the inner cortical enzymes such as 17-hydroxylase, 11-beta hydroxylase and 21-beta hydroxylase, whilst 3-beta HSD activity particularly in the human foetal and adult adrenals is low (Hornsby et al., 1984). The hypothesis of steroid gradients determining function depends on the stimulation by ACTH under normal conditions having a tonic influence on homeostasis, but also has important effects under chronic changes. During long term growth of the adrenal by ACTH, the zonal widths alter, the zona fasciculata increases and the zona glomerulosa decreases to maintain a constant adrenal mass. ACTH stimulates glucocorticoid synthesis but not aldosterone since cAMP levels are increased thus depressing p450 CMO activity. An increase in steroid production also depresses phosphodiesterase activity (Schmidtke et al., 1976) and thus the zona glomerulosa width decreases and the zona fasciculata increases. Conversely, in sodium depletion in which it becomes necessary for an increase in aldosterone production, an increase in AII levels and possibly K^+ levels will stimulate mitogenesis (see above) and in the case of AII, will increase phosphodiesterase activity and reduce cAMP levels, both of which will enhance p450 CMO activity whilst limiting p450 11-beta and 17-hydroxylase activity (Crivello and Gill 1983.,

Hornsby and O'Hare 1977). This will then increase the width and secretion rate of aldosterone in the zona glomerulosa at the expense of the zona fasciculata. This movement in zonal function and width is central to the concept that all adrenocortical cells are essentially the same but the level of activity of the enzymes in the zones over a period of time are controlled by the substance(s) present in the vasculature gradient.

2.11 NEURAL INNERVATION

It has been well recognised that the adrenal medulla has pre-ganglionic sympathetic innervation since myelinated nerve fibres have been visualised running from the splanchnic nerve through the adrenal and directly innervating the medulla. However, the existence of a postganglionic^{para}/sympathetic nerve supply to the cortex as well as medulla is now gaining credence. At the level of the electron microscope, Unsicker 1971 was able to locate nerve fibres in the perivascular space independent of blood vessels but in close proximity to cortical cells in all zones of the rat, pig, man and golden hamster, which suggested a general mammalian characteristic of adrenal innervation. However in Unsicker's studies, dense cored vescicles and synaptic membrane specialisation with vescicles, were rarely observed, although neurotubules and/or filaments were numerous. This weakened the idea of neural innervation, but rather a theory for sensory fibre involvement was postulated by Nijima and Winter in 1968, suggesting the existence of baro- and chemoreceptors in the adrenal gland.

Despite these early studies, others such as those carried out by Vogt (1944), had shown that in hypophysectomised dogs, infusions of epinephrine caused an increase in cortical secretion and in at least half of these experiments, electrical stimulation evoked cortical steroid secretions. Dallman (1977) as described in an earlier section, indirectly showed a hypothalamic-adrenal connection via crossover afferent and efferent nerve fibres.

In 1961 and 1965 Mikhail and coworkers extended the investigation into neural innervation and its localisation, to observations made at the light- microscope level in adult mammals and pre- and post pubertal rats to compare innervation in the developing zona reticularis. Distinctive para- and sympathetic innervation was specifically localised to the medulla, whereas the cortex was solely innervated by the parasympathetic system. This appeared to be densely innervated within the zona reticularis (when it developed) and the inner cortical zones. Migally 1979 has corroborated this with early reports by Lever (1953) and Kiss (1951), of nerve plexuses in the zona reticularis and zona fasciculata, as well as the presence of a subcapsular plexus cited in the rat adrenal gland. Robinson et al., 1977 showed that there were dense nerve plexuses in the zona reticularis of sheep, whilst Uno (1977) found the same pattern of distribution in the monkey.

At the electron microscope level, the contents of the neural elements including the size and density of the vesicles contained within the fibres, has made it clear that more than one type of neurotransmitter may be secreted. It is believed that catecholamines are confined to

small (60nm) dense vescicles, whilst the larger vescicles are thought to contain substances such as substance P or vasoactive intestinal peptide (VIP). In relation to the identity of the neurotransmitter substance, Robinson et al., 1977 working on sheep adrenals was able to positively identify catecholaminergic fibres by fluorescence and more recently catecholaminergic fibres independent of splanchnic derivation, have been positively identified in the rat adrenal cortex (Klietman et al., 1985). These fibres appear to be specifically localised to the outer cortex and this may be important in identifying the possible neurotransmitter involved since in the past ten years several neurotransmitter substances have been identified in the adrenal cortex. An example was the immunohistochemical staining for vasoactive intestinal peptide (VIP) (Hökfelt 1981) throughout the rat cortex in comparison to the sparser localised catecholaminergic innervation. Other contenders involved in neural transmission have included substance P, a transmitter of primary neurons and found localised both in adrenal medullary cells in the rat and in nerve vescicles in the rat and human adrenal cortex and capsule (Kuramoto et al., 1985, Linnoila et al., 1980). Neuropeptide tyrosine (NPY) a 36 amino acid originally isolated from porcine brain and widely distributed in the mammalian central nervous system and periphery has often been found associated with norepinephrine and is also found in the adrenal neural elements in the rat cortex (Varndell et al., 1983) and may be co-stored with norepinephrine and potentiate any effects. Another catecholamine previously thought to be a neural factor in the adrenal cortex is dopamine. Dopamine and its receptors of type 2 and

1 have been identified in the splanchnic nerve (Missale 1985.,1986) and demedullated and denervated adrenals have been shown to have depleted stores of norepinephrine (NE) and epinephrine (E) in the medulla, whereas dopamine concentration did not change markedly suggestive of a cortical origin of dopamine. De novo synthesis was not required since an inhibitor of catecholamine synthesis, - methyl- p tyrosine, did not affect dopamine levels (McCarty et al., 1984). Despite the presence of dopamine receptors in the rat, bovine and most recently in human cortical adrenal cells, (Dunn et al., 1981., Bevilacqua et al., 1982, Missale et al., 1985, 1986, Stern et al., 1986) it has not been possible to localise dopamine to cortical neural elements. In contrast, Shima et al., 1984 discovered functional catecholaminergic- cAMP receptors of beta-2 type receptors in the glomerulosa cells in the rat, which suggests that norepinephrine may be the important neural transmitter within the cortical nerve fibres. It cannot be overlooked that Robinson et al., in 1977, identified cholinesterase neural cell types in the sheep adrenal cortex, and high affinity muscarinic type M1 cholinergic binding sites have been found in bovine cortical tissue compared to nicotinic cholinergic sites in bovine and rat medulla (Hadjian et al., 1982, Yamanaka et al.,1986). In hypophysectomised and medullectomised rats, Kolta and coworkers (Kolta et al.,1981) showed that a peripheral cholinergic pathway could also, in part, be responsible for regulating adrenal cortical function. Their studies demonstrated that administration of a cholinergic agonist, physostigmine, increased corticosterone secretion in medullectomised

rats. In support of this, adrenal re-innervation studies carried out by Engeland and colleagues (1982), using whole adrenals from neonatal rats (1 day old), transplanted to the anterior chamber of the eye demonstrated innervation of cholinergic fibres - monitored by choline transferase activity - in contrast to intact control adrenals. They concluded that cholinergic fibres may be responsible for adrenal re-innervation and specifically supply non-chromaffin tissue. Despite sparse innervation as described above, many cortical cells contact with their neighbours with characteristic nexuses so that neurotransmitters released locally into the extracellular spaces may impinge on several cell processes thereby amplifying the effects. Catecholamines whether released from nerve fibres or cortical or medullary cells, may interact with one another in controlling steroidogenesis, or the sensitivity of the adrenal gland to glucocorticoid release. Racz et al., (1984) indicated a possible catecholamine- dopamine interaction; studies done on cultured adrenocortical cells, showed that prior incubation in cell medium containing catecholamines or medullary tissue, caused dopamine to produce inhibitory effects on steroidogenesis, whereas incubation without medullary tissue produced dopaminergic stimulatory effects. Thus there may be an interaction between neural, cortical and medullary tissue in the overall control of steroid secretion (see summary and conclusions, Chapter 12 for further postulates of serotonin- catecholamine interactions). Other theories as to the importance of neural innervation have focused on the possibility that neural elements are involved in controlling adrenal sensitivity and

pulsatility to ACTH stimulation. Ottenweller et al., 1978,1982, studied the daily rhythms of plasma corticosteroid secretions in hypophysectomised and control rats and also in rats transected at level T-7 in the spinal cord. The rats were given ACTH implants and the plasma corticosterone rhythmicity was seen to be disrupted by spinal cord transection , which suggested to the authors that innervation was important in the response rhythm to ACTH. Since then however, corticosterone rhythms in denervated rats have been detected, so its importance in this respect is debatable. Neural innervation may also be of some importance in maintaining blood flow to the adrenal gland, particularly in the haemorrhaged state. Engeland and coworkers showed that in conscious dogs undergoing slight haemorrhage, the adrenal responsiveness to ACTH was increased in splanchnectomised dogs, to offset the decrease in ACTH delivery as a result of less neurovascular regulation of the blood supply (Engeland et al 1985).

Chapter 3

STEROIDOGENESIS

3.1

STEROIDOGENESIS

From 1935-1942 twenty seven steroids were isolated from the adrenal glands of cattle. There are species differences in the number and importance of steroid products, for example in the rat all steroids are produced by all zones of the adrenal gland except the mineralcorticoid, aldosterone which is exclusive to the outer cortical zone. The other well recognised 18 hydroxylated products including 18 -hydroxydeoxycorticosterone (18-OHDOC) and 18-hydroxycorticosterone (18-OHB) which also have a degree of mineralocorticoid activity are products particularly of the inner zones in all species studied including the rat, ox, man, bird and reptile. Present evidence indicates that in vivo in man 18OHB always accompanies aldosterone secretion whereas in the ox, 18-OHB secretion is independent of aldosterone. Similarly, New World monkey and Antarctic seal adrenal cells in vitro produce 18-OHB without aldosterone. In superfusion studies using isolated rat capsular and decapsulated portions, Tait and coworkers (Tait et al., 1970) demonstrated that the inner zones specifically produce corticosterone (B), 18-OHB, and 18-OHDOC whereas the capsular portions specifically produce 18OHB and aldosterone.

3.2 BIOSYNTHETIC PATHWAYS

Adrenocortical cells are the most studied system for steroid biosynthetic pathways and the majority of data originates from mammalian studies using tissue homogenates, adrenal slices and

isolated cells to follow the activity of the steroid hydroxylating enzymes by measuring absorbance spectral changes, or using high specific activity radiolabelled steroid precursors to follow the fate and production of steroid products. Steroid hormones are produced in minute amounts and cholesterol is the precursor for their synthesis. Cholesterol can be synthesised from a series of condensation reactions with 2C acetate molecules within the adrenal tissue, or blood borne cholesterol from hepatic or dietary origin can be taken up via low density lipoprotein receptors located on the adrenal, and esterified to unsaturated fatty acids within intra-adrenal lipid droplets before utilisation in steroid biosynthesis. The proportion of adrenal and plasma cholesterol utilisation varies from species to species, e.g. in man 80% of the cholesterol is derived from peripheral sources and of this only 0.1% is necessary to meet full steroidogenic requirements.

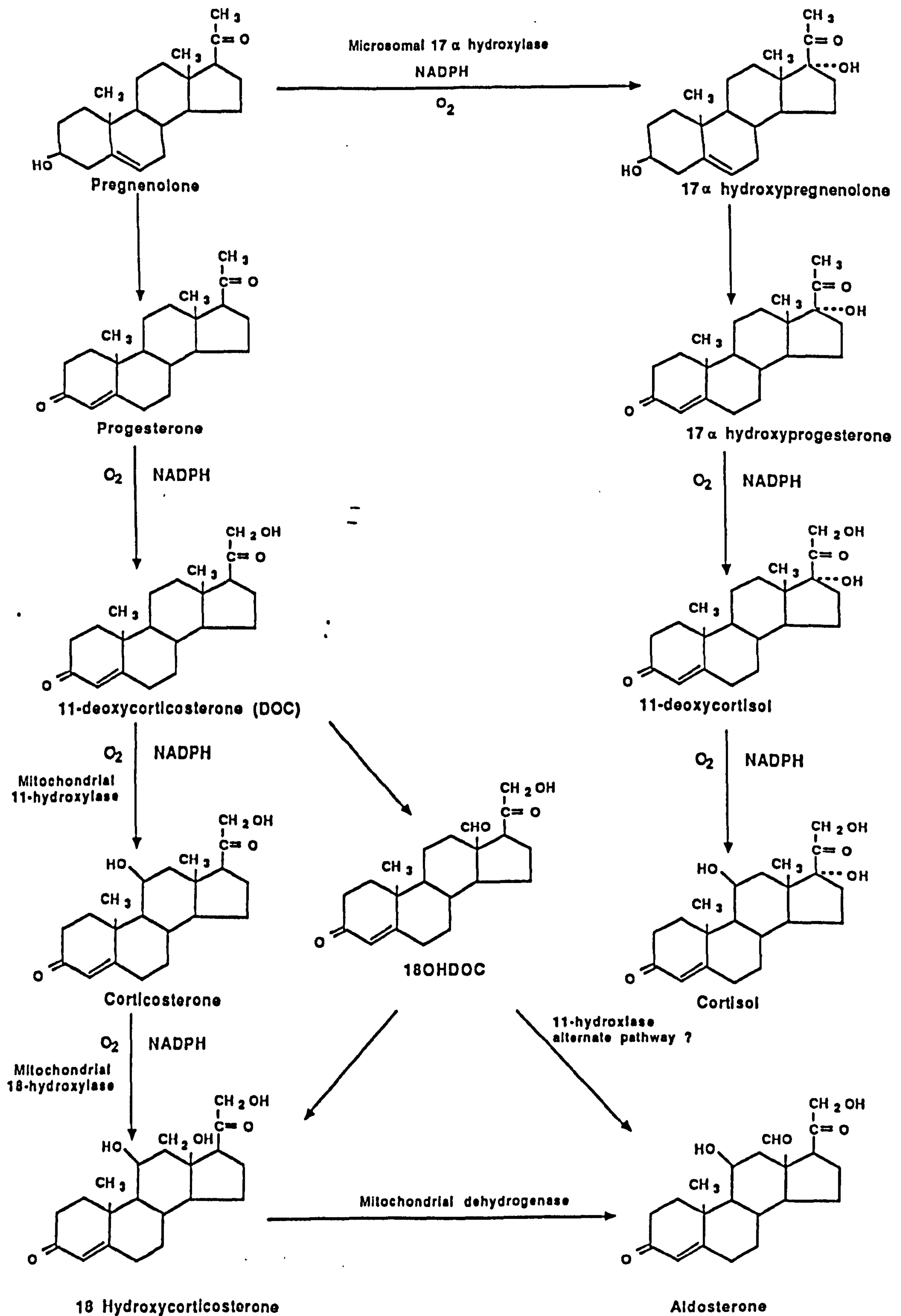
Free cholesterol is formed by the action of cholesterol esterase acting on cholesterol ester within the lipid droplet. It may then be transported to the mitochondria by one of two postulated mechanisms. In the first it has been observed that treatment of ACTH stimulated cells with a microtubule/microfilament inhibitor of polymerisation of subunits, such as cytochalasin B, prevents transport of cholesterol to the mitochondria. Another postulate is that cholesterol is ferried to the mitochondria via sterol carrier proteins (SCP) which have been described in adrenal, ovary and testis tissue. At this point, it may be pertinent to refer to the in vitro work of Whitehouse and Vinson (1971,1975) and Vinson and Whitehouse

(1969, 1973,) in which they postulated that aldosterone production in particular within the zona glomerulosa of species such as the rat, reptile and teleost fish, may occur by binding of steroid precursors before pregnenolone formation, to protein moieties (see below). The control point for steroidogenesis is considered to be the side chain cleavage of cholesterol to pregnenolone and a 6C fragment isocaproic aldehyde. The cleavage enzyme, or desmolase, exists as an organised complex, and is one of a group of four distinct forms of cytochrome P450 enzyme complexes that have a characteristic absorption spectra at the 450nm wavelength. The cytochrome P450s are a large and diverse family of enzymes found in invertebrates, bacteria and yeast. Each cytochrome system utilises molecular oxygen and NADPH as cofactor and because an adequate supply of oxygen is needed, the adrenal gland is equipped with a rich blood supply, this is particularly important during ACTH induced stress in which the blood supply to the adrenal is enhanced to increase steroid output. With the use of electron microscopy and immunohistochemical staining, the cytP450 side chain cleavage complex (cytP450scc) has been located on the inner mitochondrial membrane facing the mitochondrial matrix. Like other mitochondrial cytochrome P450 complexes, the other components include an FAD containing flavoprotein (called NADPH adrenodoxin reductase), a cytochrome P450 haemoprotein, and an Fe²-S₂ iron-sulphur protein, adrenodoxin or testodoxin, depending on its tissue of origin, all of which are located within the inner mitochondrial membrane and matrix. Thus the mitochondrial membrane system is a limiting factor in the transfer of extramitochondrial

cholesterol to the inner mitochondrial site of cleavage. In acute studies using ACTH to stimulate steroidogenesis it was found that prior treatment with cycloheximide prevented side chain cleavage of cholesterol. Cycloheximide inhibits the transcription of messenger RNA into protein, thus a labile protein factor was postulated as responsible for transferring cholesterol across the mitochondrial membranes to the active site of cytP450_{scc}. More recent studies have identified the putative protein in ACTH treated cell cytosol fractions, as a large precursor molecule of 28 kilodaltons (kDA) which was readily induced by ACTH to increase cholesterol to pregnenolone production when added to cycloheximide pre-treated cells (Pederson and Brownie 1983). Disruption of mitochondrial membranes obviates the need for the labile protein in ACTH induced steroidogenesis (Jefcoate et al., 1974) however, it appears that cholesterol transport may not always occur by labile protein transport since in AII stimulated steroidogenesis the early pathway is not inhibited by cycloheximide (Kramer 1980). Another possibility pertains to the requirement for membrane mobility and flexibility and since AII stimulated steroidogenesis may influence phospholipid membrane turnover and mobility (see section on AII stimulated steroidogenesis), it may be that AII exerts a direct effect on mitochondrial membranes, obviating the need for the labile protein required in ACTH mediated steroidogenesis.

In 1956 cholesterol intermediates were first isolated with the initial oxidation product identified as 20(S)-20-hydroxycholesterol, followed by production of (20R,22R)20,22 dihydroxycholesterol an

Fig 3.1 Main pathways for steroid biosynthesis in adrenal including aldosterone and alternate pathway



unstable intermediate that decomposes to pregnenolone. The whole reaction sequence requires 3 moles of molecular oxygen and 3 moles of NADPH for every mole of pregnenolone produced. Pregnenolone appears to be the precursor of all C21 steroids, and follows two alternate routes, depending upon the product: (i) through progesterone \rightarrow 17- α -hydroxyprogesterone (17 α -OH prog) \rightarrow 11-deoxycortisol (DOF) \rightarrow cortisol (F); or (ii) progesterone \rightarrow 11-deoxycorticosterone (DOC) \rightarrow corticosterone (B) \rightarrow aldosterone.

The major routes in steroid production are shown in Fig.3.1 and are described in detail below.

The conversion of pregnenolone to progesterone was first demonstrated in 1951 to occur via two closely linked steps: (i) oxidative dehydrogenation at position 3 of pregnenolone and (ii) transferral of the 5-4 double bond to position 4-3. The former reaction requires 3-hydroxy steroid dehydrogenase and the latter reaction requires the enzyme 5-4- isomerase both of which are bound to smooth surfaced membranes within the microsomal fractions. Thus pregnenolone within the mitochondrial membranes is transported to the cell cytosol for conversion to 17-hydroxypregnenolone or progesterone. In the former case, 17-hydroxypregnenolone can be further hydroxylated to cortisol in mammals including man and cattle, but also within the inner zones of the adrenal, can be converted to androgens and oestrogens; 17-hydroxyprogesterone is also sequentially hydroxylated to cortisol and both reactions are irreversible. The sequence of hydroxylation is not strictly rigid, but in most studies pertaining to man and the rat, the hydroxylation occurs sequentially at position C21, C11 and



latterly C18 for the production of 18-hydroxylated steroids and aldosterone. However, hydroxylation can precede reduction and 21-OH pregnenolone can act as a precursor for 11-DOC and B, whereas 17- α -21- pregnenolone can give rise to 11-DOF. The pathway to aldosterone production is not wholly defined and will be discussed in a later section.

In rats the pathway for B production - the main corticosteroid product of all zones - is most often studied, and precursor studies have delineated the pathway for synthesis. Hydroxylation at position C21 of the progesterone molecule, occurs by the action of the microsomal localised enzyme, 21- hydroxylase, which unlike the mitochondrial cytP450 enzyme complexes, does not contain the haemoprotein. The product, 21-OH progesterone, is transported by as yet, an unknown mechanism, to the inner mitochondrial membrane to be further hydroxylated at position C11 by the cytP450 11 - β - hydroxylase. The enzyme complex contains the three components described for cytP450_{sc} although the absorption spectrum is slightly different allowing for differentiation of the two enzyme complex forms. It is capable of both 11- and 18- hydroxylation and in this regard, may play an important regulatory role in 18-OHDOC formation and subsequent aldosterone secretion as described by Vinson and Whitehouse (1973,1975,) and Fattah (Fattah et al., 1975,1976,1977). The primary precursor, DOC is converted to corticosterone in all zones of the adrenal and Vinson and Whitehouse have postulated that it is formed independently of aldosterone by association of free precursor steroids whereas aldosterone and its precursors including

the 18- hydroxylated derivatives, are formed via protein bound intermediates. These theories arose from experiments with zona glomerulosa and fasciculata cells, using dialysis to measure the amount of free versus bound labelled steroid products in ACTH and cortisol pre-treated cell preparations. Their results showed that B is preferentially released whereas aldosterone and 18-OHDOC appears to be non-dialysable and thus bound until stimulated by ACTH to release aldosterone into the medium. Following the discovery of 18-oxygenated steroids the mitochondrial 18- OH- dehydrogenase complex has been described in all zones of the adrenal gland, however until the enzyme can be solubilised and isolated the exact mechanism of 18-hydroxylation remains undefined.

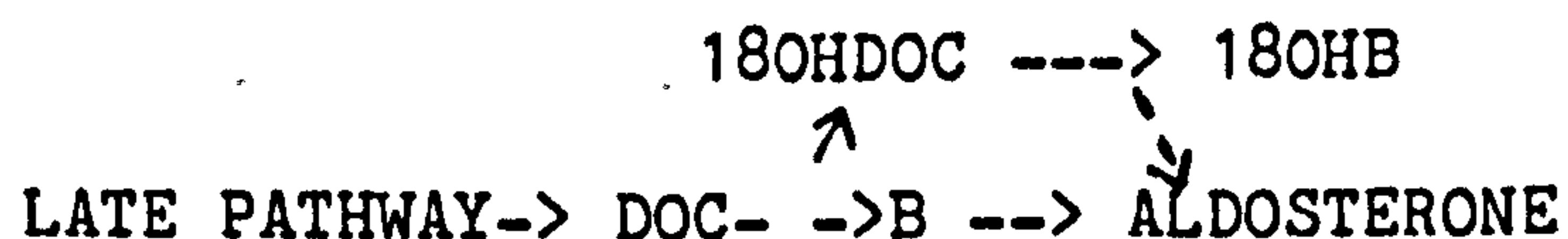
3.3 BIOSYNTHESIS OF ALDOSTERONE

Classically, the biosynthesis of aldosterone was thought to follow the reaction sequence cholesterol-> pregnenolone -> progesterone -> deoxycorticosterone -> corticosterone -> aldosterone. In 1960 Ulick and Solomon (see Idelman 1978) found that in amphibian adrenal slices 18 - hydroxycorticosterone (18OHB) always accompanied an increase in aldosterone secretion, and a similar finding was observed in other species such as the rat, man, bird and reptile. In 1967 Greengard and coworkers (Greengard et al., 1967) studied the transformation of B by bullfrog adrenal mitochondria and found by measuring the carbon monoxide (CO) induced inhibition of 18- oxygenation, that

cytochrome P450 was essential for both the conversion to aldosterone and 18-OHB. The implication from these studies was that B was a necessary precursor for aldosterone production and required 18-hydroxylation before conversion to aldosterone. However, Grekin in 1973 incubated human tumour and normal tissue with 18-hydroxydeoxycorticosterone (18-OHDOC) and found that tumour tissue in particular, could easily convert 18-OHDOC to 18-OHB and aldosterone and postulated an alternative precursor to B in aldosterone biosynthesis. Whitehouse and Vinson (Vinson et al., 1969 and Whitehouse et al., 1971), showed that cortisol pre-treatment of rats, inhibited the production of B from capsular adrenals, without affecting DOC, 18-OHB and aldosterone production. Further, labelled precursors such as acetate entered the endogenous steroid pool and labelled end products were produced when stimulated by ACTH (Whitehouse and Vinson 1973), whereas precursors after pregnenolone did not mix with endogenous steroid pools and were freely dialysable unlike the endogenous steroids. Cortisol pretreatment blocked the activity of 11- β hydroxylase, and all 18- oxygenated products were not dialysable, indicating the presence of tightly bound steroids in the alternative pathway for aldosterone biosynthesis. ACTH administration lifted this restraint and allowed free conversion of DOC to free B. In this regard they postulated that ACTH may have a late pathway effect in stimulating the release of DOC from bound components in 11-hydroxylation (see section on ACTH stimulated steroidogenesis). The existence of more than one pool of steroid has been described for the rat, bovine and guinea pig, as well as in

rat ovarian tissue.

In a series of studies in adrenal capsular portions from control and sodium depleted rats, Fattah et al 1975,1976,1977 demonstrated the preferential conversion of labelled steroid precursors to 18OHDOC and aldosterone, independent of an increase in 18OHB, with the differential suppression of B synthesis, particularly during low sodium status. The pathway is delineated below:



In more recent studies using duck adrenal slices it was found that the availability of B rather than 18-OHB was the limiting factor in the reaction kinetics for aldosterone production; In AII stimulated aldosterone production Haning (1970) and Aguilera and Catt (1979a), noted that the rate of conversion of B to aldosterone was increased by AII whereas 11-DOC to aldosterone was not, and studies by Fraser et al in 1981 showed that mitochondrial enzyme activity of 11- β hydroxylase was not affected by AII stimulated steroidogenesis. Salt depletion per se may also have an effect on late pathways(Fattah et al 1975.,1976.,1977) as may ACTH induced or cortisol blocked steroidogenesis as demonstrated by Vinson and Whitehouse (see above).

3.4 EFFECTS OF STIMULI ON EARLY AND LATE PATHWAY FOR ALDOSTERONE BIOSYNTHESIS

3.4.1 ACTH

It is well recognised that ACTH stimulates the early pathway of steroid biosynthesis in acute studies. The rate limiting step in steroid biosynthesis lies between cholesterol side-chain cleavage and pregnenolone formation. ACTH stimulates cAMP formation and it is this activation that is responsible for the early events in ACTH induced- steroidogenesis. Initially Haning et al, 1970 and Muller et al, 1966 were able to show that labelled precursors were preferentially converted to B by ACTH, in an in vitro system. Vinson and Whitehouse and coworkers (Vinson et al, 1969; Whitehouse et al, 1971.), further showed that ACTH stimulated conversion of early precursors such as acetate and cholesterol to B and possibly interacted with later biosynthetic products such as DOC, in stimulating aldosterone secretion. The effect of ACTH includes a cAMP mediated phosphorylation and activation of a labile protein since prior treatment with cycloheximide in ACTH stimulated cells, inhibited cholesterol conversion to pregnenolone (Pederson and Brownie 1983). The increase in side - chain cleavage of cholesterol has been studied in isolated mitochondria in beef and rat adrenal homogenates and is paralleled by increases in heat generated type I and pregnenolone-induced type II absorbance changes in cytochrome P450 enzymes. Late pathway effects of ACTH were first described by Müller et al, 1966 using [³H]-corticosterone as exogenous precursor.

Williams and coworkers in 1972 also showed that ACTH induced late pathway conversion of B to aldosterone. However, it was not fully clarified whether in fact the availability of B, rather than a direct effect of ACTH (as well as the other stimuli tested) on the late pathway enzymes, was responsible for the effect. Metabolic inhibitors of the early and late pathways have been used to dissect out the effects of the stimuli on each part of the biosynthetic machinery. Cyanoketone acts on the early events inhibiting the conversion of cholesterol to pregnenolone, and aminogluthimide is used to inhibit the conversion of pregnenolone to aldosterone. In 1979 Aguilera and coworkers, (Aguilera et al 1979a) studied the effects of both drugs in isolated dog and rat adrenal glomerulosa cells, and in both species, ACTH stimulated the early pathway conversion when the late portion was inhibited, whereas only in the rat was ACTH stimulatory on late alternate pathway. This alternate late pathway could not be inhibited with aminogluthimide. A protein synthesis inhibitor, cycloheximide, inhibits AII induced aldosterone production (Kramer et al 1980) at a late step, i.e. after pregnenolone, it was postulated by Kramer that the stimulus induces the de novo synthesis of a protein (possibly that postulated by Vinson et al., 1969), which promotes the association of B and/or DOC with cytochrome P450 18-hydroxylase, and increases 18-hydroxylase activity. Puromycin, another protein synthesising inhibitor, also inhibits the late pathway conversion of B to aldosterone in the rat, whereas in the dog the effects are only present in the early pathway, so there may also

be species variation in protein induced steroid secretion at different loci of the steroidogenic pathway (Davis 1968).

3.4.2 Angiotensin II(AII)

Previous studies have suggested that AII has a dual effect on the steroidogenic pathway to promote aldosterone output. Muller (1966) and Kaplan (1965) were the first to show an early pathway effect of AII in the conversion of labelled cholesterol to pregnenolone. More recent work in rat, dog and bovine adrenal tissue (Williams et al 1972., McKenna et al, 1978b., Aguilera et al, 1979a) showed that AII could stimulate the late pathway conversion of B to aldosterone in in vitro systems. Blair West (Blair-West et al, 1970) showed an in vivo late pathway effect on aldosterone production in sheep, and Aguilera in 1975 also showed a late pathway effect of AII in vivo in the dog. However, as Kramer in 1980 indicated, conversion of B to aldosterone is not associated with an increase in 18- hydroxylase activity over a short exposure to AII, unlike the increase in cholesterol side chain cleavage enzyme activity, which is induced within 5 minutes, thus the late pathway induction effect of AII may only be of significance in chronic exposure to AII. In this respect, chronic exposure to ACTH or alternatively hypophysectomy, specifically increases or decreases respectively, the steroid cytochrome enzyme activities, and chronic exposure to AII may act similarly. Kramer indicated that AII acts to increase the rate of side chain cleavage and is not inhibited by cycloheximide, in the same way as ACTH induced side chain cleavage reactions, thus the mechanism in increasing cholesterol side chain cleavage by association with the cytP450 complex, differs

with respect to de novo protein synthesis in the early pathway.

3.4.3 Salt Depletion

It was first shown in vivo that simultaneous decrease in sodium (Na^+) and increase in potassium (K^+) ion concentration in the fluid perfusing the adrenal gland, could increase aldosterone secretion, however Davis observed a similar effect by decreasing Na^+ concentration alone in perfused dog adrenal gland (Davis et al, 1968). Ganong (Ganong et al, 1965) observed an increase responsiveness of adrenal tissue to AII and ACTH in Na^+ deficient dogs, as did Muller 1965 in isolated rat adrenal zona glomerulosa cells when rats had been on 2 week Na^+ deficient diets. In vitro studies with labelled precursors indicated to Müller et al, 1966, that sodium ions exerted an early pathway effect on steroid biosynthesis. The effects of Na^+ per se are difficult to separate from the changes in plasma and extracellular fluid volume in most in vivo studies, since the renin-angiotensin- system is activated in low salt status acting on aldosterone biosynthesis via an early and late pathway effect. However Boyd (Boyd et al, 1971) observed an increased conversion of B to aldosterone in Na^+ depletion and/or K^+ infusions over two days, and in studying isolated rat adrenal mitochondria Marusic in 1967 showed that rats in short term (1 day) low sodium status, achieved a greater conversion of B to aldosterone (162%), increasing to 239% and 242% by the second and fourth day respectively, as well as an increased ability to convert B to 18-OHB. Williams et al, 1972 did not observe this additional end product and postulated that this was

because 18-OHB was tightly bound to the enzyme complex or endogenous protein, before conversion to aldosterone and its subsequent release. Saruta and colleagues (Saruta et al., 1972) found that Na^+ depletion had a late pathway effect in isolated bovine zona glomerulosa. It may be that Na^+ may have a stimulatory effect on 18-hydroxylase (Aguilera et al., 1980) and/or 11 hydroxylase activity.

3.4.4 Potassium (K^+)

Over the past couple of decades it has become increasingly clear that K^+ exerts early and late pathway effects both in vivo and in vitro. Muller's early in vitro studies using labelled precursors (Muller et al., 1966) showed K^+ to have an early stimulatory influence, which has been supported by more recent studies in rat and bovine cells in isolated pathway experiments carried out by Aguilera et al., 1979a and McKenna et al., 1978a. In the latter instance the effects of K^+ on the early steroidogenic pathway were apparent between 0-6mEq K^+ /l. Tait (1980a) demonstrated that B could activate its own conversion to aldosterone in the presence of albumin in the incubation medium for several stimuli. It was discovered that the output of B was proportional to aldosterone such that the ratio Aldosterone/B was directly proportional to the B output. However it was considered that aldo/B^2 compared with B, would reveal more specific effects in the late pathway conversion, and Tait (1974) showed that 5.3 - 5.6mMK $^+$ was the only stimulus capable of inducing a late pathway conversion to aldosterone, which resulted in an increase in the ratio (A/B^2) compared with B alone. Müller in 1980 also showed a specific late pathway conversion of [^3H]-corticosterone to [^3H]-aldosterone in

rats infused over a number of hours with a potassium chloride (KCl) solution before isolation and incubation of adrenal cells. In the converse situation of K^+ depletion in rats, Baumann (Baumann et al., 1972) observed a decrease rate of conversion of [3H]-corticosterone to 3H aldosterone and 18-OHB. Species differences also occur in that Haning (Haning et al., 1970) observed a late pathway effect of range 0-8.5mEq K^+ /l in the rat, whereas Burwell (Burwell et al., 1969) observed a late pathway effect in dog adrenal slices over a concentration range of 4 - 8mEq K^+ /l. The mechanism by which K^+ stimulates the late pathway was investigated by several groups including Greengard in 1967, who demonstrated that the cytochrome P450 was involved in the conversion of B to aldosterone. Muller's studies in 1970, and more recently by Meuli and Muller in 1985, showed that in rats repleted for 48 hours with K^+ after a two week potassium deficient diet, mitochondrial cytochrome P450 absorbance changes were greater for B-induced type I absorbance changes as well as 18-OHDOC induced absorbance changes compared with 18-OHB induced changes. Thus, it was postulated that 18OHDOC may be a better precursor for K^+ induced aldosterone biosynthesis. Müller et al., 1980, also provided evidence to suggest an alternative pathway for aldosterone production from isolated rat studies in the conversion of 18OHDOC and 18OHB to aldosterone, which was dependent on Na^+ and K^+ restriction.

3.4.5 Serotonin (5HT)

There have not been extensive investigations into 5HT's early and late pathway effects in steroidogenesis, although in vitro studies are more prevalent than in vivo work. Müller (Müller et al 1965, 1967, 1968) and later, Haning in 1970, showed that in rat isolated zona glomerulosa cells, 5HT has early pathway stimulatory effects in aldosterone biosynthesis. In the first studies relating to the effects of rat serum on increasing B to aldosterone in rat adrenal slices, Müller demonstrated an early pathway effect and labelled precursors such as [³H]-pregnenolone and [³H]-progesterone were not readily converted to aldosterone by rat serum. Later identification of the rat serum compound as 5HT (Müller et al, 1968) and more recently by Mendelsohn (Mendelsohn et al, 1981), led to the postulate that 5HT stimulated aldosterone biosynthesis at the step between cholesterol and pregnenolone formation. A late pathway effect has also been confirmed in the work of Williams, using a high albumin medium (Williams et al, 1972).

Chapter 4

THE CONTROL OF ALDOSTERONE SECRETION

4.1

CONTROL OF ALDOSTERONE SECRETION

Aldosterone is the most important and potent steroid in the animal kingdom in regulating salt balance. It is secreted most probably by simple diffusion across the cell membrane of the zona glomerulosa cells in the outer adrenal cortex although another hypothesis discussed by Whitehouse and Vinson (1971) was described in chapter 3, and postulates a role for steroid binding protein(s) in the biosynthesis and secretion of endogenous steroids.

In all animals under normal ionic conditions, aldosterone appears to be under multifactorial control. The most reviewed and certainly some of the most important regulators are ACTH, AII, K^+ , and Na^+ balance. Other possible contenders in the regulation of aldosterone have included 5HT and to a lesser extent other indoleamines; over the past ten years or so prostaglandins, and peptides including atrial natriuretic factor (ANF), somatostatin, α -MSH, pro- γ MSH, β -MSH, γ -MSH, and β -lipotropin as well as dopamine, have all been implicated in the control of aldosterone secretion and will be discussed below.

4.1.1 The Renin-Angiotensin System (RAS)

Angiotensin II is a potent octapeptide which is formed when the enzyme renin, is released from the kidney and acts on a circulating plasma α 2-globulin to form angiotensin I(AI). This molecule is biologically inactive but on passing through the lung, is converted by enzymatic cleavage, to AII. Thus, under normal circumstances the

plasma renin levels parallel the AII levels and both are increased when the blood supply to the kidney is reduced in sodium depletion. Infusions of AII in vivo in man, resulted in an increase in plasma aldosterone levels (Ames et al., 1965) and 18-hydroxycorticosteroid, but not the other corticosteroids. Similar studies with AII infusions in vivo have been done for example, in sheep and dog at very low doses of AII (10 - 40ng/ml) (Mulrow and Ganong 1961., Blair-West 1962., Ganong 1965) all of which, showed an increase in plasma aldosterone. In the rat, the role of AII in aldosterone control has been debated since in vivo studies done on rats in sodium balance, (Marieb et al., 1965., Kinson et al., 1968) suggested that AII was not an important regulator. Aguilera (Aguilera et al., 1978a) impressively demonstrated that in the rat, the effects of AII were of primary importance in low sodium status - infusions of captopril (an ACE inhibitor) into rats on a low sodium diet, blocked the compensatory rise in plasma aldosterone, indicating the requirement of AII (see later section on sodium status). An alternative experiment using prolonged low dose AII infusions into conscious dogs or rats, produced the same effect in increasing the responsiveness of the adrenal and by extrapolation, AII receptor affinity and number, in the salt depleted state (Aguilera et al., 1978b, 1980). Further studies pointed to the important role of Na^+ status on the sensitivity and number of AII receptors depending on the Na^+ intake over a time scale of two weeks. It was seen that in rats, AII receptor affinity and responsiveness was increased on low Na^+ diets in rats and the converse was true for high Na^+ diets after 36 hours.

However after 4 days and longer, the affinity of AII receptors and the initial sensitivity of the response to AII, returned to control values, although the receptor numbers had increased on low Na^+ and decreased on high Na^+ diet. Hauger (1978) further showed that AII is able to regulate its own receptor number as a consequence of changing circulating levels of AII by his studies of infusion in conscious rats. Over a number of years, in vitro studies using isolated zona glomerulosa cells, have shown stimulation of aldosterone by AII, particularly in the rat model (Haning et al., 1970., Albano et al., 1974., Tait et al., 1976., Bing and Schulster 1977., Aguilera et al., 1979., Fredlund et al., 1975). Amongst others, the Tait group (Tait et al., 1970., 1976) was able to show a specific and more potent response of zona glomerulosa cells to AII in cells incubated in medium containing 5.6mMK^+ compared to 3.6mMK^+ . In both cases, the response was significant at doses of AII as low as $2.5 \times 10^{-10}\text{M}$ plateauing at $2.5 \times 10^{-8}\text{M}$. The rat plasma concentration of AII has been estimated at around $5 \times 10^{-11}\text{M}$, thus the sensitivity in vitro, is in good agreement with the physiological levels of the peptide. Whereas the Tait group achieved an 2.6 fold increase in aldosterone in vitro with AII, Douglas et al., 1976a incubating the cells in medium 199 (M199) achieved a 6-7 fold increase in aldosterone. However, the maximal response to AII was only half that achieved with K^+ or 5HT at maximal stimulatory doses (for the effects of incubation medium Campbell et al., 1982 see chapter 6). Although it is well known that renin in the kidney is responsible for maintaining the circulating levels of AII, Doi (Doi

et al., 1984) was able to measure renin present within the adrenal gland of normal and nephrectomised rats. Ryan et al., in 1967 had also located renin in rabbit adrenal glands and further, had found a higher concentration of renin in the sodium deficient rabbits as well as in nephrectomised rabbits. This was located specifically in the zona glomerulosa layer rather than the zona fasciculata and medullary zone. This has led some investigators to suggest that adrenal renin may act as local regulator of aldosterone release, particularly in e.g nephrectomised rats and dogs in which aldosterone levels are still maintained despite removal of the kidney (Palmore 1967,1969., McCaa et al., 1974). Indeed, there is a positive correlation between adrenal renin and adrenal aldosterone which would support such a concept.

4.1.2 Angiotensin III (AIII)

Angiotensin III is a metabolic breakdown product of AII. It is a heptapeptide with the Aspartyl N- terminal removed by the adrenal aminopeptidase enzyme. In man, infusions of AIII have resulted in raised plasma aldosterone levels (Carey et al., 1978) although it was shown in Carey's study that AIII had only 15-30% the steroid activity and 11-36% the pressor activity of AII in human subjects; it was postulated that AIII receptor mediated effects are through different receptor types in the smooth muscle preparation compared to the adrenal tissue. However, its physiological role in humans is not thought to be important due to its very low circulating level. In rats the circulating levels of AIII are higher than AII and, studies using isolated adrenal rat zona glomerulosa tissue have shown

a stimulatory effect specifically on aldosterone secretion (Goodfriend and Peach 1975); AIII receptors have been identified in the rat adrenal cortex (Devynck et al., 1977). Aguilera and Catt (1979b) showed however, that AIII has only 10% the potency of AII and this potency decreased with incubation time in isolated cells due to its rapid metabolism to smaller inactive peptide products. In vivo, the potency of AIII relative to AII is more difficult to assess since the metabolic clearance rate (MCR) of aldosterone also has to be considered (Messerli et al., 1977). Angiotensin III may bear more importance in pathophysiological states, such as primary aldosteronism, as in vivo infusions into man have demonstrated (Carey et al., 1979., Zager et al., 1982) an increased aldosterone sensitivity to AIII at lower doses compared with normal subjects.

4.1.3 Potassium (K⁺)

Potassium ions were first noted to specifically stimulate aldosterone secretion by Giroud (1956) using isolated rat adrenal slices. However the doses were supraphysiological and since then, it has been well established that raising the body's potassium by dietary intake, systemic or local adrenal infusion, increases the aldosterone secretion or excretion rate. For example, K⁺ loading in rats (Boyd et al., 1971) caused a marked increase in the conversion of corticosterone to aldosterone in isolated adrenal capsules. Early in vivo studies in man (Johnson et al., 1957) showed that K⁺ depletion was associated with a delayed and diminished rise in urinary aldosterone during Na⁺ depletion. Several in vivo studies in dogs,

sheep and rats have pointed to an interrelationship between K^+ stimulation of aldosterone and its trophic effects on the width of the zona glomerulosa being dependent on AII, ACTH and sodium levels in the plasma (Pratt et al., 1982., Mazocchi et al., 1985., Douglas et al., 1978) and in in vitro studies (Fredlund et al., 1977) the responsiveness to both ACTH and AII were dependent on the K^+ concentration of the incubating medium. Baumber (1971) suggested that intracellular K^+ was possibly the primary factor in the control of aldosterone secretion, but the theory was based on studies using whole adrenals. Using isolated cells, Mendelsohn (Mendelsohn et al., 1975) found that manipulation of intracellular K^+ levels or inhibition of the influx of K^+ with ouabain, did not affect the aldosterone biosynthesis induced by physiological stimuli. Potassium levels are subject to variation in several instances including postural changes, sex differences and even mild muscle activity. Insulin administration and/or depletion of glucose can alter K^+ and aldosterone levels in the plasma (Fraser et al., 1979). What is known is that plasma K^+ levels probably provide the best indicator of K^+ status, but its action at the level of the adrenal is not wholly known. However, chronic studies carried out by Douglas (Douglas et al., 1976b) showed that infusions of potassium chloride (KCl) over 1 to 6 weeks into rats on normal Na^+ status altered the number of AII receptors without an increase in PRA and hence AII levels; further studies over three days of KCl infusions (Douglas et al., 1980) showed that AII receptor affinity was increased by increasing plasma K^+ concentrations (c.f. the work of Aguilera et

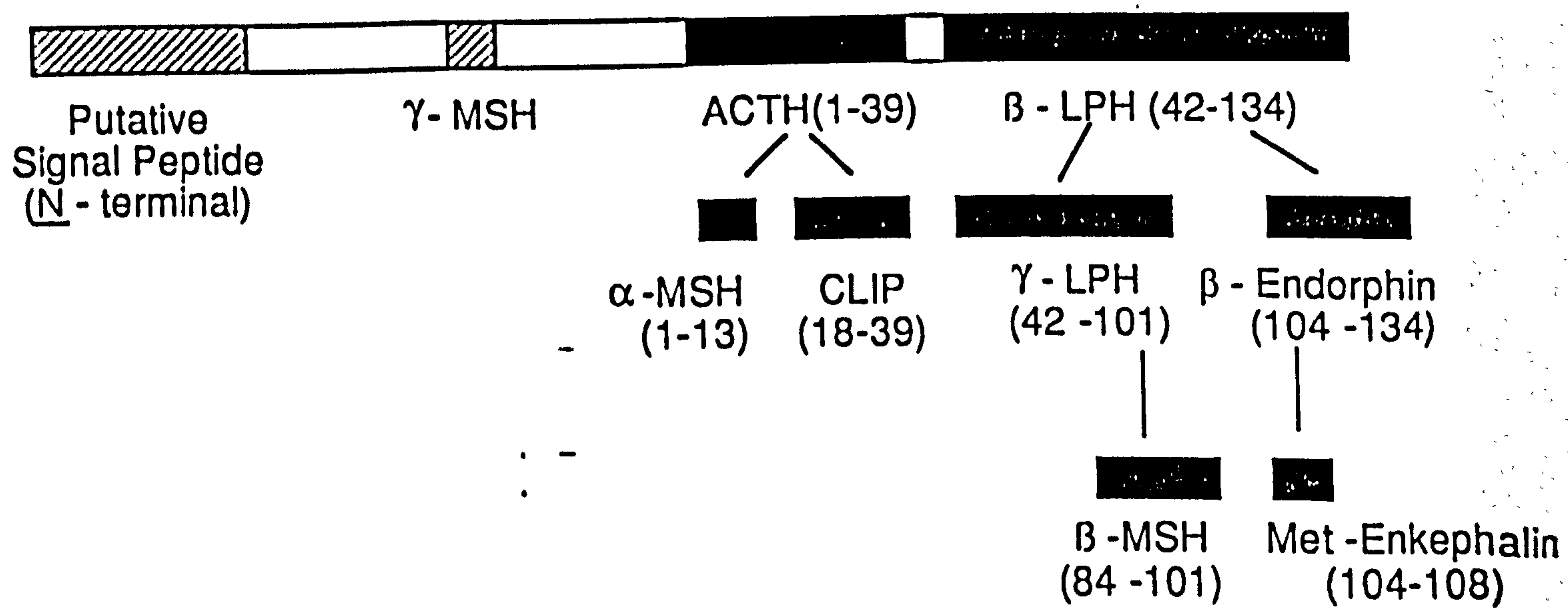
al,1978b for Na⁺ status and AII receptor characteristics).

Although Na⁺ depletion had previously been shown in rats to affect the secretion rate of aldosterone, and increase the width of the zona glomerulosa as well as the conversion of corticosterone to aldosterone a similar finding was shown with K⁺ loading (Dean et al,1948, Boyd et al., 1971, Mazzochi et al., 1985). With the exception of 5HT, K⁺ is the only known stimulus specific to the zona glomerulosa cells (Tait et al., 1972, Haning et al,1970, Albano et al., 1974). The effects of increasing K⁺ concentrations in vitro have been extensively investigated by Haning, Tait, and Bing and Schulster (Haning et al., 1970, Tait et al., 1972, Bing and Schulster 1977). In these studies the maximal aldosterone secretion was repeatedly obtained at 8.4mMK⁺ with further doses being inhibitory. Other groups including Fakunding (1979) using isolated cell systems incubated in M199, have found a plateauing of the potassium response at 13 - 15mM K⁺. Braley and Williams found maximal aldosterone secretion at 18mMK⁺, whereas Marusic, found 12mMK⁺ to be the optimum concentration for aldosterone secretion (Braley and Williams 1977, Marusic et al., 1967). Although these levels of K⁺ are not achieved in vivo the significance of the aldosterone response to K⁺ being dependent on the composition of the incubating medium are interesting and will be discussed later.

4.1.4 Adrenocorticotrophic Hormone (ACTH)

Many studies both in man and rat, sheep, ox, dog and duck have shown that acute infusions of ACTH stimulates the secretion of aldosterone

Fig 4.1
POMC derived peptides : mRNA precursor molecule



(amino acid numbers in parenthesis)

whereas long term administration of ACTH in sheep for example (Blair-West et al., 1963) has either no effect, or an inhibitory effect on aldosterone secretion. ACTH is thought to act as a permissive factor in the control of aldosterone, for example in hypopituitary patients the responsiveness to other regulators of aldosterone is attenuated by ACTH removal (Williams et al., 1971). Similarly in hypophysectomised rats the in vitro responsiveness of the zona glomerulosa cells was not as great in normal sodium status compared to control, and was even more blunted in the Na⁺ deficient state. (Palmore et al., 1969, 1970, Müller et al., 1976.). In vitro studies by Tait (Tait et al., 1970, 1976,) showed that in static and perfused adrenal glomerulosa cells ACTH was able to acutely stimulate aldosterone secretion whereas the effects on perfused zona fasciculata cells were more prolonged. Mulrow et al., in 1961, showed that in haemorrhaged dogs the removal of ACTH by hypophysectomy also removed the augmentation of aldosterone secretion.

A hypothesis has been presented that ACTH by increasing the blood flow to the adrenal arteries and ramifying capillaries, causes increased vascularisation with time, and it is this increase in blood supply that causes cellular hypertrophy as opposed to hyperplasia. It is well recognised that ACTH is derived from a large precursor peptide molecule (POMC) produced in the pituitary gland of several animal species, however the relative importance of POMC-derived peptide in the control of aldosterone secretion is not yet fully

established (see Fig. 4.1). β -lipotropin and β -MSH both stimulate aldosterone secretion in the rat (Matsouka et al., 1981) although Pederson et al., 1980a using synthetic porcine β - LPH did not find any stimulatory effect of this, and several other pituitary fragments including, α MSH, synthetic human β - endorphin. In vivo β -endorphin specifically stimulated aldosterone secretion in the hypophysectomised-nephrectomised dog, without affecting cortisol levels (Gullner et al., 1983). The fact that higher concentrations of β -endorphin are present in the dog pituitary compared to the rat may point to a species specific concentration dependent variability that in turn dictates its importance in aldosterone secretion; β -endorphin has also been found in the monkey, and beef pituitary glands.

Vinson et al., 1980 showed convincingly that α - MSH had a marked stimulatory effect in isolated rat zona glomerulosa cells. It was also pointed out that the levels of α -MSH are elevated in the pars intermedia region of the rat pituitary in salt depletion, so that its physiological role may be of importance in varying sodium status. However Whitehouse (Whitehouse et al., 1982) could not show a change in α -MSH levels in rats on low salt diets, although the in vitro sensitivity (defined in this paper as a change in the lowest dose of peptide required to elicit a response) of zona glomerulosa cells to the peptide was increased (Vinson et al., 1983). The circulating concentration of α -MSH in rats has been estimated at 10^{-10} M. In normal rats this is not enough to stimulate corticosterone from the

zona glomerulosa and inner cortical zones; however in the Na^+ deplete rat the sensitivity of the aldosterone response to α -MSH approaches the circulating levels, so that the low Na^+ state increases the sensitivity of the adrenal zona glomerulosa to this peptide in vitro as shown by Vinson et al., 1981, 1983 and in vivo in hypophysectomised rats as demonstrated by Shenker et al., 1985. In the latter case α -MSH specifically increased plasma aldosterone levels to normal in hypophysectomised rats compared to intact rats on low salt diets. They postulated that α -MSH may in part be involved in regulating aldosterone levels during low salt status since low salt also increases the concentration of α -MSH in the pituitary. That α -MSH may act through its own specific receptor was suggested by Vinson et al 1984 in studies done on isolated rat zona glomerulosa cells. Using a series of synthetic analogues to α -MSH, including [Nle⁴,D-Phe⁷]- α -MSH, and the naturally occurring peptide fragment γ -MSH, they were able to show that α -MSH unlike the other two peptides, increased aldosterone secretion by interaction with a receptor of differing steric requirements to the other peptides. However more recently Baumann 1986 showed that α -MSH was not specific to the zona glomerulosa and using a series of α -MSH and melanotropic peptides suggested that the Phe⁷,Tryp⁹ sequence, which is common to these and the ACTH molecule, may indicate an interaction with a low affinity ACTH receptor, which would be in keeping with Vinson's findings that α -MSH at 10^{-5}M increases cAMP, possibly through interaction with the ACTH receptor.

Beta LPH (β -LPH) is the other major peptide fragment derived from the precursor molecule POMC, and can be further degraded to several peptides including γ -LPH (42-101), and β -endorphin (104-134). Gamma LPH can be further digested to β -MSH (84-101), and met-enkephalin within the β -endorphin sequence. Both the β - and α -MSH peptides, share a common amino acid sequence, and may have some potentiating effects on aldosterone biosynthesis. Notably, both peptides show an enhanced responsiveness to salt depletion in the rat (Vinson et al., 1983, Yamakado et al., 1982). Beta LPH was first isolated from ovine pituitary glands and Matsouka (Matsouka et al., 1980) showed that rat capsular cells as opposed to decapsular cells, were stimulated to secrete aldosterone by human and sheep derived β -LPH (10^{-9} M- 10^{-7} M). Corticosterone was also stimulated in capsular preparations but the effects were not cyclic AMP mediated, thus not all pituitary fragments appear to stimulate through ACTH-cAMP coupled receptors.

The combined molecular weight of ACTH and β -LPH only accounts for 1/3-1/2 the molecular weight of the whole precursor mRNA sequences, but Nakanishi in 1979 also identified an amino acid sequence occurring at the N-terminal portion of the pro-hormone, preceeding the ACTH sequences and followed by basic amino acid sequences involved in a common peptidergic cleavage reaction. It was named γ -MSH and is contained within a 16 kilodalton (kDa) fragment. Gamma MSH was identified and sequenced from extracts of bovine pituitaries using mRNA to probe the amino acid sequences (Nakanishi et al., 1979)

and the naturally occurring peptide was also identified in frozen human pituitary glands as a 77-amino acid fragment and called pro- γ -MSH. Pederson et al., 1980a, 1980b, incubated isolated rat zona glomerulosa cells with several peptides of ACTH derivation including a 16kDa fragment derived from the N-terminal end of POMC. Only the 16kDa fragment was able to stimulate aldosterone secretion when co-incubated with ACTH. Although it was originally thought this effect could be due to ACTH contamination, Al-Dujaili et al., 1981a using superfused zona glomerulosa cells from the rat, was also able to show that pro- γ -MSH could potentiate the response to ACTH, but the cells needed to be primed with the peptide before the potentiating effect was seen. They argued that this was due to the prerequisite stimulation of protein synthesis. By adding actinomycin to the cells beforehand, the potentiating effect of pro- γ -MSH was lost. Pederson had previously shown that in stressed rats another hormone apart from ACTH was responsible for the activation of cholesterol ester hydrolase, and later work showed that the 16kDa fragment in hypophysectomised rats in vivo, was able to activate this enzyme. It remained for Pederson (1980b) to synthesise a γ -MSH with a further 15 amino acids added to the C-terminal region that acted identically to the 16kDa fragment in potentiating the effects of ACTH on aldosterone secretion in vivo in the hypophysectomised rat model. In human adenoma tissue the γ_3 -MSH peptide also potentiated the effect of ACTH in vitro (Lis et al., 1981). Pederson most recently (Pederson et al., 1986) used isolated human aldosteronoma cells to further

investigate the mechanism of aldosterone secretion in the zona glomerulosa cells. The aldosterone response is very sensitive to doses as low as 10^{-12} M γ -MSH and measurement of free cholesterol levels within the cytosol have given further credence to the suggestion that γ -MSH activates cholesterol ester hydrolase (Pederson et al., 1986).

More recently however, Griffing et al., 1985 has measured the levels of pro- γ MSH in IHA patients, and found a positive correlation with aldosterone levels and pro- γ -MSH levels. Despite in vitro evidence that pro- γ -MSH can specifically potentiate the effects of ACTH in isolated rat adrenals (Al-Dujaili et al., 1981) the role of this and other ACTH precursor molecules in essential hypertension and IHA is not clear. Further, in Carey's study (Carey et al. 1984) dexamethasone treatment did not prevent the rise in plasma aldosterone in IHA, which would suggest that MSH of pituitary source at least, was not involved. Despite this, it must be pointed out that other sources of these various peptides have been localised to the pars-intermedia and it is known that in the rat, there is both serotonergic and dopaminergic input to this area that may regulate the release of the peptides.

Beta LPH (β -LPH) was shown by Matsouka et al., 1981, to increase aldosterone secretion in rat zona glomerulosa cells together with β -MSH (part of the β -LPH sequence). However neither peptide caused any apparent increase in cAMP levels. β -MSH contains the heptapeptide sequence (Met-glutamic acid(Glu)-histidine(His)-

phenylalanine (Phe)-arginine(Arg)-tryptophan(Tryp)-glycine(Gly)-
 common to ACTH and this may explain the peptide core sequence
 requirement for aldosterone stimulation in some species. In the
 same in vitro rat studies, Matsouka 1981 was not able to show a
 stimulatory effect for β -endorphin, a peptide fragment derived from
 the C-terminal of the β -LPH molecule. β -endorphin is
 pharmacologically the most potent endorphin and in hypophysectomised-
 nephrectomised dog it had a potent stimulatory effect in perfused
 adrenals (Gullner and Gill 1983), whilst the smaller cleavage product
 derived from β -endorphin, met-enkephalin, had no stimulatory effect
 in the dog or rat (Matsouka et al., 1981). There may be species
 differences in the potency of these peptides. In this regard both
 α - and β -MSH have been shown to have mitogenic effects in foetal rat,
 rabbit, sheep and human adrenal tissue; for example, Challis et al.,
 1977 showed that in rabbit foetal tissue there was an increased
 sensitivity to α -MSH when the foetal adrenal was not responsive to
 ACTH, which gradually declined as ACTH responsiveness increased.
 These changes were paralleled by a changing content of MSH in the
 pituitary as described above. γ -LPH, which is derived from β -LPH, is
 also thought to have aldosterone stimulating properties. It is
 derived from the β -MSH sequence as opposed to the β -endorphin
 sequence, which in the rat has no stimulatory effect.

4.1.5 Sodium Status

A correlation between Na^+ status and aldosterone secretion was first observed by Leutscher and Axelrad 1954 and is now well established in a variety of animals. How Na^+ acts to alter aldosterone secretion has not been completely elucidated. In vivo studies have shown in man, dog, sheep and rat a parallel activation of the RAS system during Na^+ depletion and the opposite effect during Na^+ loading (Douglas et al 1978b, Aguilera et al., 1978a, Blair-West et al., 1963, Davis 1968) and as indicated above there is also an interrelationship between Na^+ status and K^+ concentration. Williams et al., 1971 tried to isolate the effects of Na^+ ion changes from K^+ changes in the blood and study the effects of these changes on the response to AII and ACTH infusions in man. It was suggested that Na^+ principally controls and maximises the sensitivity of the adrenal response to AII and ACTH and more recently Dawson-Hughes 1981 demonstrated, again in man that the plasma aldosterone response to AII, was steeper in low Na^+ status compared to the effects of prolonged low dose infusions of AII, in concordance with the earlier work by Oelkers et al., 1975; thus, indicating that in addition to the change in sensitivity to AII, Na^+ per se has a potentiating effect.

Although AII does have an important role in Na^+ status there are reports of an increase in aldosterone levels in low Na^+ conditions without an increase in renin and AII e.g. in the nephrectomised rat and dog studies (McCaa et al., 1974, Pratt et al., 1981).

Aguilera 1980 also showed that in low Na^+ status (4 days), plasma aldosterone, side chain cleavage enzyme activity, 18-hydroxylase activity and AII receptor number in the rat model, were more markedly increased than circulating AII levels alone in normal Na^+ balance, indicating a potentiating effect of Na^+ on all these parameters. Hypophysectomised- nephrectomised dogs on low Na^+ diets, did not have a compensatory increase in plasma aldosterone compared to nephrectomised dogs with intact pituitary glands (McCaa et al., 1974). It has also been inferred by studies done in man, that Na^+ per se or factor(s) activated in low Na^+ status (possibly pituitary), may interact with the RAS system in increasing the response to AII infusions. Oelkers and coworkers (Oelkers et al., 1975) and Dluhy (1972), showed that in man, prolonged low dose infusions of AII in normal Na^+ states , only partially reproduced the effects of bolus infusions of AII in increasing the sensitivity of the plasma aldosterone response in low Na^+ status. In clinical studies patients with panhypopituitarism were unable to increase plasma aldosterone on low sodium diets compared to control patients although the RAS system remained intact (Williams et al., 1971). This suggested that other factors as well as AII are important in low sodium status.

4.2 OTHER POSSIBLE LOCAL AND HUMORAL FACTORS

4.2.1 Aldosterone Stimulating Factor (ASF)

Aldosterone Stimulating Factor (ASF) is a relatively newly identified glycopeptide of anterior pituitary origin. Originally it was isolated in human urine extracts (Sen et al., 1981a,) and found to have potent aldosterone stimulating properties in vitro in a variety of species including rat (Sen et al., 1981b), cat and rabbit. It may also be responsible for the potentiating effects of low sodium in nephrectomised dogs and rats with an intact pituitary (McCaa et al. 1974, Palmore et al., 1969 see above).

In the clinical syndrome, idiopathic hyperaldosteronism (IHA) a tentative role for ASF has also been suggested; Biglieri et al., 1984 suggested that the increased responsiveness and thus elevated levels of aldosterone could be related to an increase in ASF in the pars intermedia. Patients with IHA had elevated ASF levels compared to control and adrenal hyperplastic patients .

4.2.2 Atrial Natriuretic Factor (ANF)

Early work by Anderson et al., 1959 pointed to an inhibitory effect of atrial extracts on aldosterone secretion in the dog. More recently, Atarashi et al., 1984 using rat atrial extracts, showed that the extract did indeed inhibit basal aldosterone secretion in in vitro rat adrenal cells and further, the dose response to AII or ACTH was inhibited. The granularity of the atrium decreases in salt loading and increases after adrenalectomy, indicative of possible

interaction between aldosterone levels i.e. increased release and inhibition of aldosterone secretion in the volume expanded/salt loaded state , and the converse in the salt depleted /low volume state, which would support the inhibitory in vitro findings. Maki et al., 1984 deduced the amino acid sequence of the atrial natriuretic factor using complementary DNA (cDNA) techniques; the precursor peptide consists of 128 amino acids. The molecule is trypsin sensitive, since treatment with the enzyme abolishes it's inhibitory effects.

There are two peptides, Atriopeptin I and Atriopeptin II of 21 and 23 amino acids respectively, which are derived from the precursor molecule 20,000-30,000 kDa. Chartier et al., 1984 and Maki et al., 1984 both showed that synthetic ANF (Arg101-Tyr126) later called ANF 48-73, were the important regions of the peptide in producing inhibitory effects on the dose response to AII, K^+ and ACTH, in rat zona glomerulosa cells. Studies of isolated bovine adrenal cells and superfused adrenal glands have shown no effect of ANF on basal aldosterone production, and only a slight inhibition of the AII dose response (Frisina et al., 1986) Like other studies, these conflicting results may be a consequence of species differences and/or the cell system used to investigate the effects. In the pathophysiological state, an interesting observation has most recently been made by Cappuccio et al., 1986 who demonstrated that in normal subjects loaded with high salt, and then administered a mineralcorticoid analogue, fludrocortisone, sodium retention

increases in the first 24 hours after administration of the steroid. Thereafter, the 'escape' phenomena in which Na^+ excretion equals Na^+ intake despite high mineralcorticoid plasma concentrations over the same time period, is associated with an increase in circulating ANF levels. They suggested that ANF may be important in augmenting this natriuretic 'escape' effect both in this experimental model and in patients with hyperaldosteronism where the escape effect is relevant. ANF receptors have been identified in crude rat adrenal capsular preparations with a high binding affinity K_A 0.1nM and low capacity, comparable to those found in the vasculature (Schiffrin 1985). Similarly, ANF binding sites have been found in bovine adrenal zona glomerulosa cells (DeLean et al., 1984a). Despite ANF's non-specific inhibitory effects on stimulated aldosterone secretion, it could not be displaced by other peptides such as AII from its binding site, or by several calcium receptor blockers, indicating that it does not act via a non-specific calcium channel to block stimulated steroidogenesis.

4.2.3 Oxytocin (OXT) and Vasopressin(AVP)

Ang et al., 1984 after having discovered oxytocin and AVP in the intermedio-lateral grey columns of the spinal cord in humans decided to look for these compounds in the adrenal medullary tissue of rat, man and human phaeochromocytoma tissue. The intermedio-lateral grey columns are the site of location for pre-ganglionic neurons in the sympathetic nervous system, including the adrenal medulla so the possibility existed that these peptides were neurotransmitters for

any post-ganglionic connections to the medulla. By the use of several techniques including radioimmunoassay (RIA), high performance chromatography (HPLC) and histochemistry, Ang was able to measure large quantities of both OXT and AVP in the adrenal. Oxytocin appears to occur in larger amounts with concentrations in the range 19.9-162.2pg/g compared to AVP of 9.8-103pg/g. Both values are markedly greater than the corresponding free plasma levels measured of 1.3-3.1pg/ml for OXT, and 1.9-3.8pg/ml for AVP, suggestive of an endogenous source for both substances within the adrenal gland. Nicholson et al., 1984 found by RIA measurement similar ratios of both compounds in human foetal adrenal tissue. Swansons group (1980), suggested that there may be a neuroendocrine link between the paraventricular nuclei of the hypothalamic region, and the adrenal i.e. possible afferent and efferent nerve fibres. Ang and coworkers working on this assumption and the role of OXT and AVP in this connection, performed electrolytic lesioning to the paraventricular nucleus (PVN) area in rats but this did not change the existing concentrations of the peptides in the adrenal. By another approach, Nussey et al., 1984 looked at the concentrations of AVP in the plasma, pituitary, adrenal and the hypothalamus of normal and Brattleboro rats. These rats are genetically different in that they do not have AVP in the hypothalamus and thus, are unable to conserve water. Despite this defect, it was found that both types of rat had approximately the same amounts of AVP in the adrenal, thus there appears to be a dissociation of PVN and adrenal contents of

AVP, which would argue against a neuroendocrine connection for this peptide at least. The possibility therefore exists of endogenous synthesis and inter-relationships with the cortex and medulla. In this regard, there have been suggestions that both peptides may influence the secretion of catecholamines from the medulla and therefore affect the secretions of the corticosteroids (see section on catecholamines). AVP can influence the metabolic turnover of norepinephrine, and oxytocin directly depresses the activity of pre-ganglionic sympathetic neurons in the spinal cord. As well as these interactions, specific binding sites for AVP have been located in a crude capsular membrane preparation in the rat (Balla et al., 1985). These receptors have high affinity and low capacity and correlating with this, isolated perfused zona glomerulosa cells treated with increasing doses of AVP (10^{-8}M - 10^{-6}M) synthesised more aldosterone over a 2 hour period, although similar concentrations of AVP in a static system had no effect (Payet et al., 1982). In 1984, Payet suggested that AVP in the long term, may act as a local growth factor since similar results were found using cultured zona glomerulosa cells, and so the possibility remains that one or both peptides may have multiple functions in the maintenance of growth in the cortical regions and the modulation of catecholamines and steroid interactions in the medullary regions (see also functional zonation of the adrenal gland).

4.2.4 Catecholamines

The adrenal medulla contains large quantities of epinephrine within its chromaffin granules and smaller amounts of norepinephrine. Norepinephrine is commonly associated with the post-ganglionic neurotransmitter in the sympathetic nervous system although both amines have wide neurotransmitter functions. Indeed, histochemical studies in both normal and transplanted sheep adrenals, have shown sparse post-ganglionic aminergic innervation which enters the adrenal gland with the supplying arteries which may represent another potential source of catecholamines in the gland. Within the adrenal gland, the catecholamines exert a regulatory influence on steroid biosynthesis and in particular, the secretion of glucocorticoids. In the emergency state, catecholamines increase the synthesis and release of glucocorticoids from the zona fasciculata and the close interaction of the cortical and medullary zones are thought to be facilitate this role. In the zona glomerulosa, both norepinephrine and several analogues including the isomers isoproterenol(-), alprenolol(-) and the (+) isomers at lower potency, are able to stimulate aldosterone secretion with an increase in cyclic AMP levels (DeLean et al., 1984b). These effects are mediated through β -1 receptor types as defined in vascular tissue, however binding sites have not been investigated to correlate with the steroid data.

4.2.5 Histamine

Histamine was found to stimulate aldosterone secretion in hypophysectomised-nephrectomised dogs by Aikawa et al., 1979, which provided some evidence that histamine may have a direct effect on the adrenal gland. More recently, Edwards et al., 1980b performed in vitro and in vivo studies in the rat and man respectively. In their in vitro studies, using isolated, perfused rat zona glomerulosa cells, the effects of histamine antagonists, rather than the agonist itself, were investigated. Using the H₂ antagonists, cimetidine and ranitidine, they were able to elevate aldosterone basal production and selectively block the aldosterone response to AII. The H₁ receptor antagonist, chlorphenhydramine, enhanced the aldosterone response to ACTH and AII. In vivo treatment of subjects with the H₂ antagonist cimetidine, blocked the aldosterone response to furosemide without affecting plasma renin levels (PRA). Both types of study suggest the possible existence of a type H₂ receptor interaction with AII receptors in the zona glomerulosa cells.

4.2.6 Prostaglandins

Prostaglandins are a series of lipid based compounds of ubiquitous nature. They are sub-divided into a several types.

Flack et al., 1969 were the first workers to show a stimulatory effect on aldosterone secretion in vitro using rat zona glomerulosa cells and type E₁, E₂ and F₁α-prostaglandins. However, Spat et al., 1975 was only able to show a stimulatory effect of the type E prostaglandins in Na⁺ depleted rats. A role for prostaglandins in

the Na⁺ deplete state has been suggested by Morise et al., 1982 in which subjects infused with indomethacin and captopril, were compared with subjects treated with captopril alone in the Na⁺ deplete state; the former group did not show an exaggerated response to ACTH compared with the control or captopril group. Thus, they postulated that prostaglandins may mediate in part, the increased responsiveness of the adrenal to ACTH in the sodium deplete state. However the results of prostaglandins do not always follow a logical pattern and indomethacin has also been shown to block the effects of AII in vitro. Recent reports have also claimed a role for type A prostaglandins in aldosterone secretion possibly augmenting the increased responsiveness of glomerulosa cells in the sodium deficient state.

4.2.7 Somatostatin

Immunohistochemical studies have shown that somatostatin exists within the zona glomerulosa and Aguilera (Aguilera et al., 1982) has recently reported the existence of somatostatin binding sites in the rat glomerulosa cells. Somatostatin inhibits aldosterone secretion in rat adrenals but not in the dog, and receptor sites have not been found in this species or in human and monkey tissue. Thus, like other stimuli the effects are species dependent. Aguilera and others have argued that it is the release of dopamine, which under certain physiological conditions stimulates the release of local stores of somatostatin in the adrenal cortex and may be the primary regulator of somatostatin release.

4.2.8 Dopamine

The dopamine antagonist, Metoclopramide (MCP), has been shown to enhance aldosterone secretion in vivo in man (Edwards et al., 1980a, Carey et al., 1976, 1980, Norbiata et al., 1977, Brown et al., 1977) on normal sodium diets without concomittant increases in PRA, ACTH or plasma K^+ and this led investigators to postulate that aldosterone is under maximum tonic inhibition by dopamine in the normal sodium state. Edwards (Edwards et al., 1980a) using infusions of dopamine in an in vitro rat cell system, showed that the stimulatory effects of MCP were specifically blocked by dopamine, whereas the AII stimulated aldosterone response was not. However, since these earlier studies, there seems to be convincing evidence that dopamine may interact with AII, in differing Na^+ states. Aguilera et al., 1984, infused AII with (MCP) in rats on high Na^+ diets and showed an increase in the sensitivity of the aldosterone response to AII in this situation, comparable to aldosterone levels found on normal salt intake. Thus, MCP appeared to derepress the possible dopaminergic inhibitory effects existent in the sodium replete state. Gordon et al., 1983, was able to show a similar derepression in humans. Conversely, infusions of dopamine in human subjects, together with AII, prevented the increase in sensitivity to AII normally seen in low Na^+ status (Edwards et al., 1975, Aguilera et al., 1984). In keeping with its possible physiological role during changes in sodium status, Alexander et al., 1974, found that urinary dopamine excretion increased in sodium repletion within minutes of an

increase in extracellular volume and Carey et al., 1981, found a decrease in plasma and urinary dopamine in sodium depletion. Dopamine receptors have been found in rat (Dunn et al., 1981) tissue, and have even been subclassified as type D2 in rat and D1 in calf adrenal tissue (Bevilacqua et al., 1982). More recent studies using specific dopamine antagonists have been carried out by Missale et al., 1985, 1986 in which the rat dopamine receptor types have been classified as DA1 and DA2 as opposed to type D1 and D2 receptors found in the brain. Both adrenal DA receptors are cyclic AMP linked, DA1, stimulating aldosterone and DA2, inhibiting aldosterone synthesis. DA2 receptors have therefore been assigned an important physiological role in the adrenal cortex since dopamine inhibits aldosterone release in some experimental studies, both in vivo and in vitro. The origin of this dopamine is still highly speculative. Edwards (1980a) direct studies on zona glomerulosa cells and the studies by Wilson et al., 1983, in which ganglionic blockade of the autonomic nervous system did not prevent the direct effects of MCP infusions in man, suggest that in these species at least, dopamine is locally active. Indeed dopamine has been localised in the adrenal chromaffin granules of several species including the rat (Racz et al., 1974, McCarty 1984, Hannah et al., 1984), although it is also postulated that dopamine is not endogenous and is rather taken up and deconjugated of its sulphate moiety from the circulation (Van Loom 1980).

4.2.9 Serotonin (5HT)

Like potassium, 5HT specifically stimulates aldosterone biosynthesis in the zona glomerulosa. Rosenkrantz in 1960, was the first to investigate the effects of exogenous 5HT on steroidogenesis using rabbit adrenal slices. His work included reports on specific structural requirements for the 5-OH group in aldosterone secretion. Structure-activity relationships have since been carried out by Muller et al., 1968 and Al-Dujaili et al., 1981a. Muller showed that 5HT is a potent stimulus in rat adrenal cells but the potency is species dependent. The physiological importance of 5HT as a possible humoral agent in aldosterone biosynthesis was also studied by Muller et al., 1967 using isolated rat adrenal cell incubates. Rat serum induced aldosterone release, which could be inhibited by the 5HT antagonist, methysergide. In earlier work by Bakker et al., 1960, rat serum was able to stimulate aldosterone release but the effects were not completely blocked by dihydroergotamine, and it was not thought that the full effect was 5HT mediated. Similarly, Mendelsohn et al., 1981 showed a similar effect of human serum on isolated rat glomerulosa cells but the effects were greater than those due to 5HT. Groups such as Haning et al., 1970; Albano et al., 1974; Tait et al., 1972, 1975; Bing and Schulster et al., 1977 and Shaikh et al., 1984, 1986, have shown significant stimulatory effects in isolated zona glomerulosa cells. Early in vivo effects were less convincing and failure to produce significant effects on aldosterone release were attributed to uptake into blood platelets.

Since then direct stimulatory effects in both man and the rat have been reported Mantero et al., 1979, Al-Dujaili et al., 1980). Indirect evidence with important implications were reported by Maestri et al., 1985 . In this study, infusions of MCP, known to stimulate aldosterone secretion (see above), was infused after prior treatment three hours before, with a specific 5HT antagonist, pizotifen. The reasoning behind this protocol being that if MCP is acting as a 5HT agonist rather than a dopaminergic antagonist to stimulate aldosterone release, this would be blocked by the 5HT antagonist. Aldosterone alone was blocked, whilst the effects of MCP on prolactin release were intact. The possible serotonergic effects of another dopamine agonist, bromocriptine, was studied in superfused isolated rat adrenal cells by Edwards et al., 1979 who showed that the dose response to 5HT and AII could be blocked by this drug, suggesting therefore, that the effects in part of previous dopamine related drug studies may be accounted for by the 5HT involvement. A less clear cut but still significant result on the effects of ketanserin, a 5HT type 2 receptor antagonist was made by Mantero et al., 1985, in which plasma aldosterone levels in patients with idiopathic aldosteronism were reduced by administration of this drug (see also Introduction).

The case of hypersecretion of aldosterone in man in idiopathic aldosteronism is an interesting one. Originally it was subclassified with Conn's syndrome in which there is hypersecretion of aldosterone as well as adrenal hyperplasia. However, in

idopathic hyperaldosterism (IHA) there is hypokalaemia, and no significant variance in sodium concentrations in the whole body or plasma, unlike Conn's syndrome. Padfield et al., 1981, Griffing et al., 1985, and others, have pointed out that IHA is better categorised into a continuum of the essential hypertensives in which there are low renin levels and an increased sensitivity in response to AII and ACTH infusions in human subjects. Gross et al., 1981 and Mantero et al., 1985, using cyproheptadine and ketanserin, were able to show a drop in aldosterone levels in IHA, indicating some serotonergic involvement. Shenker et al., 1985 using peripheral blockers of the conversion of 5-hydroxytryptophan to 5-hydroxytryptamine, demonstrated a central involvement in man, of 5HT and/or its precursor, in stimulating aldosterone release. The essential amino acid tryptophan itself or conversion to 5HT, was shown by Modlinger et al., 1979 to increase aldosterone, renin and cortisol secretion, thereby perhaps increasing stimulatory activity of ACTH-like peptides from regions such as the pars intermedia. Indeed, Jones and co-workers (Jones et al., 1977) showed that 5HT co-incubated with rat hypothalamic tissue could selectively stimulate the release of CRF, suggestive of a possible central involvement of 5HT in adrenal steroid secretions. Corticotrophin-releasing factor, is involved in release of the large precursor molecule POMC, and has also been shown to be released by administration of 5HT precursors including 5HTP in vivo in the rat (Petraglia 1984), and this in turn increased the release of POMC peptides into the general circulation

within two hours of administration of 5HTP. The effects of 5HTP could be inhibited by the 5HT antagonist, methysergide, and lend credence to the hypothesis that 5HT may have an indirect regulatory effect through pituitary factors, in increasing aldosterone secretion (Petraglia et al., 1984). Thus, 5HT may have both central and peripheral involvement in aldosterone secretion and part of this work goes some way in determining its role in aldosterone control and secretion.

Chapter 5

RECEPTORS AND MESSENGER LINKS FOR STIMULI OF STEROIDOGENESIS

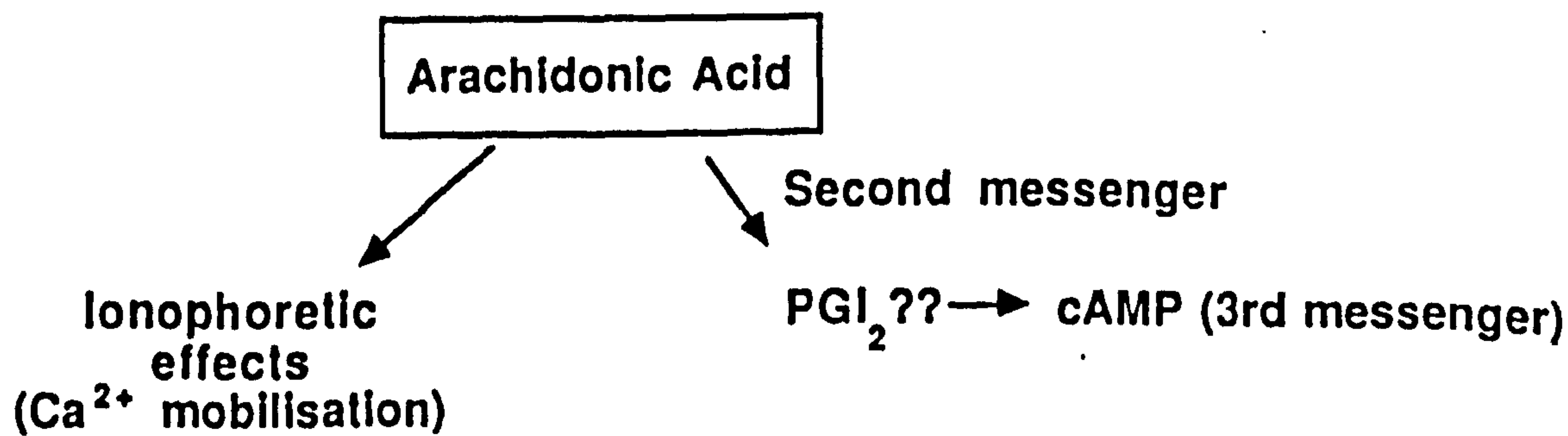
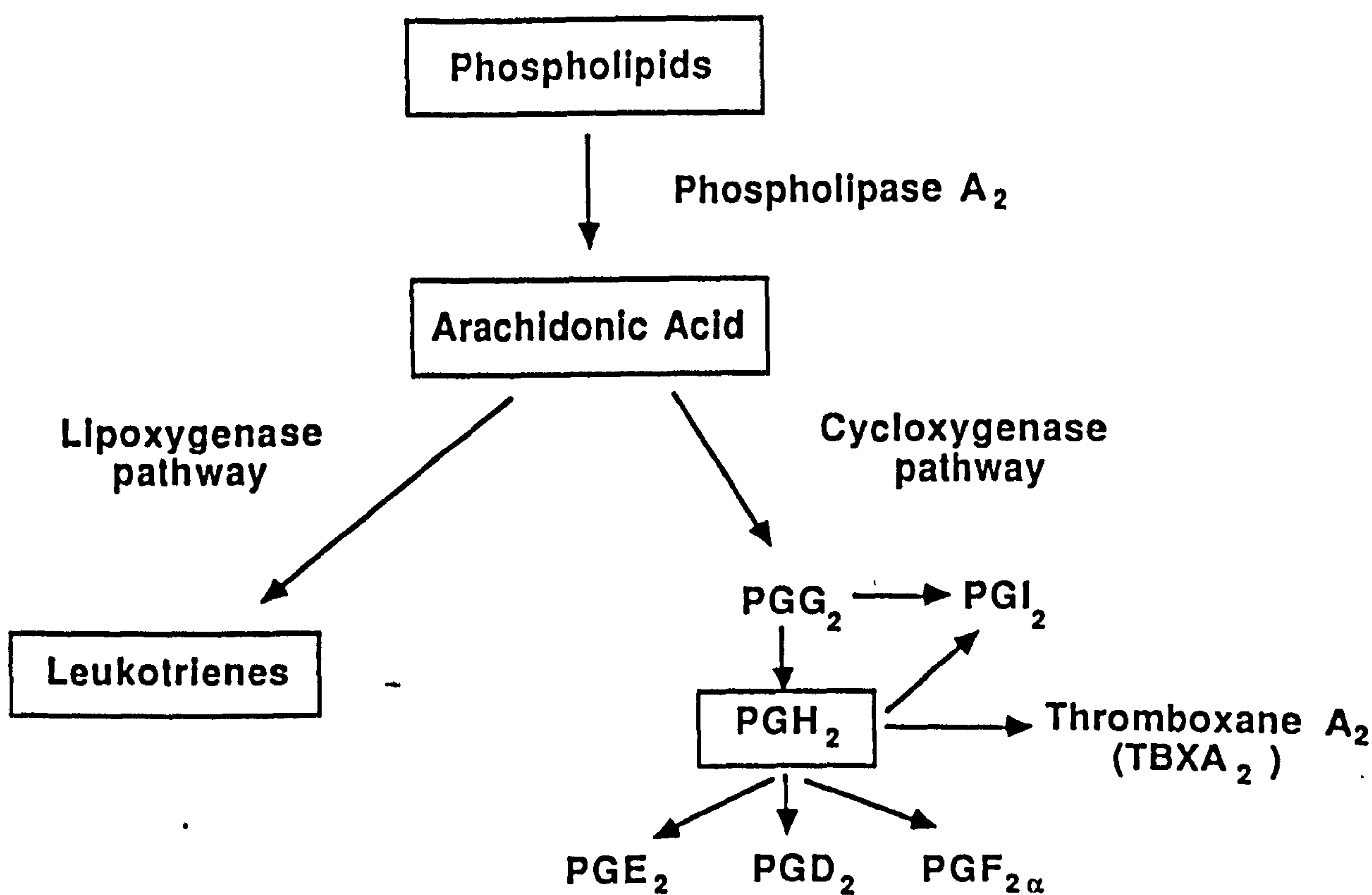
MECHANISMS OF ACTION OF ALL STIMULI IN STEROIDOGENESIS

5.1 ACTH

Schulster and Schwyzer (1980) reviewed the effects of ACTH and derivatives and concluded the sequence 1-39, in the ACTH peptide, can be divided into particular functional regions which may vary in different cell types. However it is the N-terminal amino acid sequence 1-24 which contains all the biological activity and within this subdivision sequence 1-4 is the potentiator sequence, 5-9 the message sequence which stimulates cAMP and steroidogenesis, and 11-23 the address sequence. ACTH acts on plasma membrane surface receptors exemplified by studies using ACTH molecules that were chemically linked to inert polymers thereby precluding entry into the cells (Grahame-Smith 1967), but still induced steroidogenesis. Lee et al., 1980, and Bristow et al., 1980 examined the effects of ACTH peptide fragments and antagonists including the use of a biologically inactive ACTH fragment, corticotropin inhibiting fragment (CIP)- 7-38 ACTH (Lee et al., 1980) which inhibited the steroid response at doses dissociated with the inhibition of cAMP. Bristow (1980) showed that ACTH 1-39 stimulated steroid and cAMP production, whereas ACTH 5-24 stimulated steroid output independent of cAMP. Further to this work, Bristow demonstrated a difference in inhibitory constants for the ACTH antagonist, ACTH 6-24 for ACTH 1-39 binding compared to ACTH 5-24 binding, and suggested that two receptors for ACTH exist. However, Buckley et al., 1981 suggested the existence of one type of

receptor with calcium and cAMP playing interrelating roles in steroidogenesis ; Schulster and Schwyzer (1980) postulated that cAMP at basal or even elevated levels needed to be 'guided' into physiologically important compartments for eliciting steroidogenesis. Thus, despite the early studies by Grahame-Smith (1967) indicating a role for cAMP in steroidogenesis, its primary importance as messenger in steroidogenesis has been questioned. Despite conceptual arguments that cAMP is in fact compartmentalised and that intracellular measurements would not indicate a change at lower ACTH doses, Tait et al., 1980 and others, have argued that another second messenger is involved. A major candidate for this role is undoubtedly Ca^{2+} . Birmingham (1953) was the first to propose that extracellular Ca^{2+} was needed for ACTH- stimulated steroidogenesis and others have substantiated this. Podesta et al., 1980 showed that Ca^{2+} alone under certain experimental conditions could mimic the effects of ACTH in stimulating steroidogenesis, however the Ca^{2+} ionophore A23187 only weakly increases steroidogenesis indicating that other mediators besides Ca^{2+} may be important. In this regard Shima et al., 1979 using fasciculata cells, showed that ACTH stimulated cAMP at greater levels in the presence of higher extracellular Ca^{2+} . It has been suggested that extracellular Ca^{2+} is important in the coupling of ACTH to cAMP formation, since at low Ca^{2+} concentrations the maximal response to ACTH was reduced and the ED_{50} for ACTH was increased (Fakunding et al., 1979). This was in contrast to the effects of Ca^{2+} on the ED_{50} for cholera toxin or 5HT stimulated steroidogenesis

Fig 5.1 Stimulation of Phospholipid Metabolism



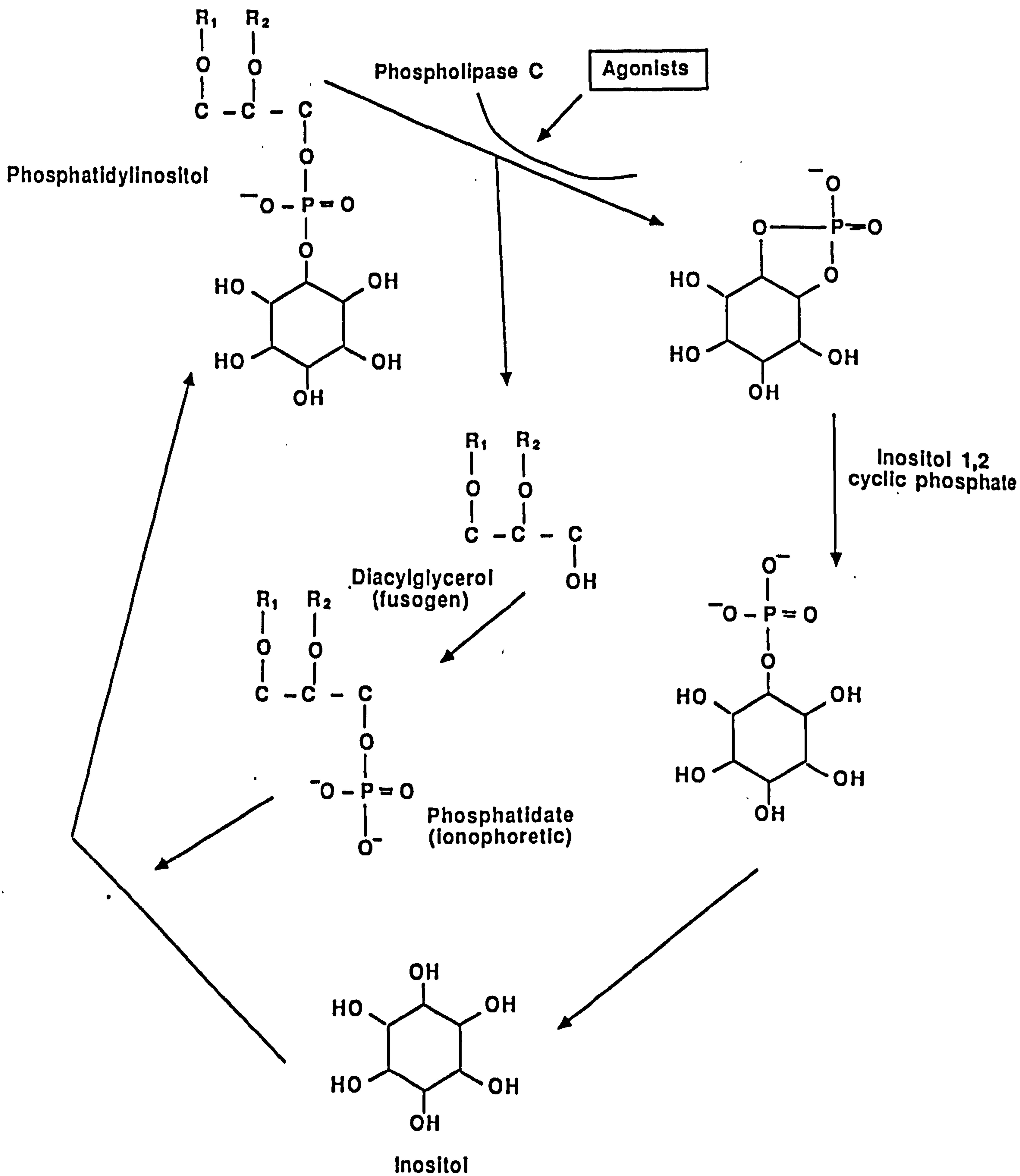
which did not change (see below). In contrast, Shima et al., 1979 suggested that intracellular Ca^{2+} was important in coupling the ACTH-cAMP-aldosterone output. An important role for extracellular Ca^{2+} was discovered by Cheitlin et al., 1985 for association and increased occupancy of ACTH receptors.

The phospholipase-prostaglandin system may also be important in mediating the steroidogenic effects of ACTH. ACTH induces a Ca^{2+} dependent increase in phospholipase A2 activity and prostaglandin synthesis formed from the precursor, arachidonic acid in the cyclooxygenase pathway. The prostaglandins include $\text{PGF}_2\alpha$ and PGE_2 both of which have been isolated in the perfusate of ACTH stimulated cat and rat adrenals or cells respectively (Laychock and Rubin, 1977). Arachidonic acid and its metabolites can either mimic or act as negative modulators of a primary stimulus. The other products of arachidonic acid metabolism are formed through the lipoxygenase pathway and are called the leukotrienes (see Fig. 5.1). Unlike the prostaglandins, they are potent ionophores and could be involved in mobilising intracellular Ca^{2+} stores in ACTH stimulated steroidogenesis. Indeed, products of arachidonic acid metabolism, and particularly the leukotrienes, have been implicated in the control of ACTH-stimulated steroidogenesis in the rat model (Jones et al., 1986).

Saruta and Kaplan (1972) reported that prostaglandins require Ca^{2+} for steroid production, and aldosterone biosynthesis was increased by prostaglandins; Morise et al., 1982 showed that prostaglandins play

a role in ACTH stimulated steroidogenesis in humans in low salt status. Cyclic AMP is also increased in response to PGA_1 , PGF_1 and PGF_2 , and provided Ca^{2+} is present, these prostaglandins increase cholesterol side-chain cleavage. The B series prostaglandins, have similar effects as the E series at lower doses, but it is well recognised that different doses have different effects. Rubin et al., 1977 had observed that PGE_2 could induce an increase in cAMP and cGMP in bovine adrenal cells and that the cGMP preceded the cAMP. This in turn preceded PGE_2 formation, which is Ca^{2+} dependent because phospholipase A_2 activity requires Ca^{2+} . There is also evidence that phospholipase A_2 and phospholipase C activity may be indirectly linked since metabolites of phospholipid turnover, stimulate phosphoinositol (PI- general abbreviation) hydrolysis and reacylation; conversely, diacylglycerol a product of PI hydrolysis, is rich in arachidonic acid and is a precursor for arachidonic breakdown products, obtained through phospholipase A_2 activity. The possible importance of this process lies in the hypothesis that arachidonic acid and reacylation may be important in altering membrane lipid dimensions and thus, affect internal Ca^{2+} mobilisation and/or adenylate cyclase activity in stimulus-secretion. Phospholipase C is also activated by Ca^{2+} and is responsible for removing the polar head group from a phospholipid and thus increases phosphatidic acid, which Farese et al., 1981,1983 in early studies, suggested as being important in steroidogenesis. Since then, new theories have been expounded, most notably by Rasmussen and Barrett

Fig 5.2 Hydrolysis of Phosphatidylinositol



(1984) in which Ca^{2+} has been implicated in two important roles, one of which included PI hydrolysis and subsequent diacylglycerol and inositol triphosphate (InsP_3) formation - see later sections. Phosphatidylinositol (PtdIns) and its phosphate derivatives, phosphoinositol 4,5 - biphosphate (PtdIns (4,5) P_2) and phosphatidylinositol 4-phosphate (PtdIns 4P) are components of plasma membranes and have a high level of turnover when stimulated by some extracellular messengers. Although PtdIns turnover may be closely linked to receptor occupancy, it may not be associated with receptor stimulation. Michell (1977) hypothesised that PI may be responsible for Ca^{2+} gating, and Rasmussen and Barrett (1984) have already stated that Ca^{2+} messenger systems may involve PtdIns hydrolysis. The products of PI hydrolysis include diacylglycerol, which is rich in arachidonic acid and is thought to play an important role in mobilising intracellular pools of Ca^{2+} not bound to mitochondrial stores (see section on AII); and other metabolites include inositol triphosphate (InsP_3) and less importantly inositol biphosphate (InsP_2). InsP_3 causes the release of Ca^{2+} from intracellular stores, in particular the endoplasmic reticulum where specific binding sites for InsP_3 have been identified; diacylglycerol modulates C-kinase enzyme activity. The C-kinase forms a branch of the Ca^{2+} -linked stimulation response and includes a series of enzymes wholly dependent on Ca^{2+} for their activity in eliciting a physiological response. Diacylglycerol may act as a fusogen in exocytotic processes (Fig 5.2) but in the steroid secreting cells

it's more important function may lie in its sensitization of protein kinase C to free Ca^{2+} levels in the cytosol, and this enzyme in turn is responsible for the phosphorylation of proteins (Takai et al., 1985). Phosphorylation of diacylglycerol with production of phosphatidic acid has also been postulated to act as an endogenous ionophore, and thus alter membrane permeability to calcium. In contrast, prostaglandins produced from phospholipid breakdown, stimulate adenylate cyclase and do not have ionophoretic characteristics. It is more than likely that the endogenous levels of prostaglandins and/or phosphatidic acid, reflect a situation where they interact with other messengers in potentiating a response. The other branch of the Ca^{2+} chain requires Ca^{2+} to modulate and activate Ca^{2+} -calmodulin dependent phosphodiesterase activity.

5.2 ANGIOTENSIN II (AII)

The intracellular mechanisms which mediate the effects of AII are uncertain, although Albano et al., 1974, suggested that AII stimulated cAMP production in the zona glomerulosa cells of the rat. Later work discussed by Tait et al., 1980, suggested that the effects of AII on cAMP were probably a result of impurities in the preparation rather than a direct result of AII. In Tait's studies (Tait et al., 1974, 1975, 1980), cAMP did not change at all doses of AII used (10^{-10} M - 10^{-7} M). Other groups, (Shima et al., 1978, Fujita et al., 1979), were not able to show an increase in cAMP levels in either the rat or dog zona fasciculata or zona glomerulosa,

over a range of doses 10^{-11} M - 10^{-5} M even in the presence of the phosphodiesterase inhibitor, theophylline. In Shima's study, levels of cAMP were reported to decrease in response to an increased phosphodiesterase activity. Calcium has been postulated as the most important, if not sole regulator, in AII steroid production. Shima et al., 1978 showed that in isolated rat cell preparations, decreasing the extracellular Ca^{2+} concentration or introducing a Ca^{2+} antagonist such as lanthanum, verapamil or tetracaine, blocked the response to AII. Fakunding et al., 1979, further showed that the decrease in extracellular Ca^{2+} decreased the maximal output to AII stimulation, without affecting the ED_{50} response and the same was seen for potassium and 5HT stimulated steroidogenesis (discussed below). AII binding was also not affected by Ca^{2+} in contrast to ACTH receptor binding (see above). Braley et al., 1984, using an intracellular calcium dye QUIN 2, was able to show a parallel increase in fluorescence of the dye with increasing intracellular Ca^{2+} concentrations, together with an increase in AII stimulated steroidogenesis. There has been speculation as to whether AII increases steroidogenesis by an increase in Ca^{2+} influx, or efflux, caused by intracellular Ca^{2+} redistribution. Williams et al., 1981, showed that pre-incubation of rat zona glomerulosa cells with ^{45}Ca followed by stimulation with AII, increased the efflux of Ca^{2+} , which would tend to suggest that AII is responsible for a redistribution of Ca^{2+} within the cell. In contrast however, Elliott et al., 1981, observed an decrease in ^{45}Ca influx into bovine adrenal zona

glomerulosa cells, but an immediate increase (2 min) in Ca^{2+} levels intracellularly, parallel with an increase in aldosterone secretion. Thus, to include all the accumulated data on Ca^{2+} mobilisation would be to include the hypothesis that AII receptor interaction controls the movement of both intra- and extracellular Ca^{2+} in the zona glomerulosa cells. It has already been seen that AII stimulates phosphodiesterase activity, possibly the reason for the lowered levels of cAMP observed in Fujita's study (Fujita et al 1979), and this enzyme activity modulation has also been related to an association of a Ca^{2+} binding protein, calmodulin, that acts as an intracellular Ca^{2+} receptor. It is thus conceivable that AII receptor interaction could increase calmodulin- Ca^{2+} binding, and this in turn could modulate Ca^{2+} dependent forms of phosphodiesterase activity (Koletsy et al., 1983). Indeed, calcium-calmodulin dependent phosphodiesterase is known to exist in the adrenal and is activated by increases in free cytosolic calcium. Hence, the levels of Ca^{2+} and cAMP could be critical and determined in AII stimulated steroidogenesis, by the tuning up of Ca^{2+} mechanisms and the tuning down of cAMP mechanisms. Most recently studies carried out by Kojima et al., (1984) demonstrated the importance of Ca^{2+} in eliciting the two phased aldosterone response (described above Rasmussen (1984)), in perfused bovine zona glomerulosa cells treated with AII. They found that the aldosterone response could be divided into an initial transient phase component that was specifically dependent on the increase in free cytosolic calcium induced by initial hormone receptor

interaction; the same acute response could be induced by addition of the Ca^{2+} ionophore, A23187 instead of AII. However, the initial interaction of AII with its receptor induced changes in PI hydrolysis caused by the activation of phospholipase C through extracellular Ca^{2+} entry into the cell. An increase in $\text{PtdIns}(4,5)\text{P}_2$ turnover occurred with the resultant increase in InsP_3 , which is important in inducing the release of Ca^{2+} from non-mitochondrial pools. As stated before, increases in free cytosolic Ca^{2+} are required for the initial calmodulin dependent transient phase of the response. The prolonged Ca^{2+} -dependent phase is dependent on the production of diacylglycerol and InsP_3 which in turn modulate and sensitize the C-kinase enzyme (present in the cell cytosol), to cytosolic Ca^{2+} , both in the initial transient phase of the response when intracellular Ca^{2+} levels are increased and later when the levels of Ca^{2+} reach a maximum and then begin to decline, but the enzyme remains sensitive and active even at the lower concentrations of Ca^{2+} , thus inducing the prolonged AII-induced steroidogenic response phase. Confirmation of this latter effect was established by looking at the effects of the compound, 12-O-tetradecanoyl phorbol 13- acetate (TPA) which when added to perfused adrenal cells alone, induced the sustained phase of the AII response curve. TPA is thought to act specifically on the C-kinase branch of the Ca^{2+} response curve (see Kojima 1984) in the same way as InsP_3 and diacylglycerol, sensitizing the enzyme to cytosolic Ca^{2+} .

5.3 POTASSIUM (K⁺)

Early in vitro experiments showed that K⁺ stimulated steroidogenesis included an increase in cAMP levels, and this occurred at higher doses e.g. at 3 - 6mMK⁺ (Boyd et al., 1976.); 3.6-6.4 mMK⁺ (Albano et al., 1974); 3 - 8.4 mMK⁺ (Tait et al., 1980) and the latter group did not observe an increase in cAMP levels at other doses of K⁺. Tait et al., 1972 also showed that cells incubated in the presence of a high dose of cAMP, and increasing doses of K⁺, showed an additive effect of K⁺ with cAMP on steroidogenesis, although this was not apparent at doses greater than 8 mMK⁺. They suggested that at lower doses of K⁺, steroidogenesis is mediated by a cAMP independent mechanism, and at 8 mMK⁺, cAMP is the common mechanism of action. In contrast, Fakunding et al., 1979, and Fujita et al., 1979, using isolated zona glomerulosa cells, did not detect an alteration in cAMP levels in response to increasing K⁺ stimulated steroidogenesis. Further, changing the extracellular concentrations of Ca²⁺ affected the maximal response to K⁺ and AII without affecting the ED₅₀ of stimulated steroid production. It was also observed in Fujita's experiments, that there was a lack of additivity in steroid output when cells were incubated with K⁺ and AII together and both sets of observations suggested, that both stimuli share a common mechanism of action involving Ca²⁺. Mackie et al., 1978, had observed an increase in Ca²⁺ efflux with increasing doses of extracellular K⁺, although in similar experiments using prelabelled calcium (⁴⁵Ca²⁺) undertaken by Williams et al., 1981, an increase in ⁴⁵Ca²⁺ efflux in

zona glomerulosa cells was not observed in K^+ stimulated cells. Despite the early attempts to include an important role for intracellular K^+ in steroidogenesis for some if not all stimuli, (Dluhy et al., 1972., Mendelsohn et al 1975), later studies by Mendelsohn et al., 1981 using isolated rat cells and measuring intracellular K^+ after stimulation, have not proved succesful in furthering this hypothesis.

The role of phosphinositol hydrolysis, or the PI response (see above) has received increasing credence as a modulator in AII and K^+ stimulated steroidogenesis. Indeed, Farese (1981) demonstrated that both stimuli increased the incorporation of ^{32}P phosphate into phosphatidic acid and phosphoinositol. It was postulated that both stimuli increase PI hydrolysis but AII stimulated steroidogenesis was more resistant to EGTA treatment than K^+ stimulated steroidogenesis, which tended to suggest differing mechanisms of action. More recent evidence has supported this theory as Kojima (1985) demonstrated using bovine adrenal zona glomerulosa cells perfused with $8mMK^+$. In these studies it was seen that intracellular Ca^{2+} levels increased upon stimulation with $8mMK^+$, causing a transient phase aldosterone response which was further modulated and sustained by an increase of cAMP levels rather than $InsP_3$ or diacylglycerol seen for AII-stimulated steroidogenesis. Thus, in this case only the calmodulin-dependent branch of the Ca^{2+} chain was involved in the initial phase, with cAMP playing a modulatory role in the latter sustained phase of the steroid response.

5.4 SEROTONIN AND RECEPTOR TYPES

The physiological role of 5HT and the existence of multiple receptor sites are controversial but need to be explained in some capacity to allow further understanding of a possible receptor-mediated steroidogenic response in the zona glomerulosa.

A wealth of both pharmacological and physiological data has accumulated that points to the existence of multiple 5HT receptor sites, which are discussed below.

In brain tissue, 5HT has been identified as a neurotransmitter and the anatomy of the major serotonergic pathways established. With the advent of labelled molecules, [^3H]-5HT and [^3H]-LSD in the first instance, have been used to differentiate 5HT receptor sites.

Binding studies have been performed using a series of agonists or antagonists for displacing the labelled molecule, in order to measure the K_I values, or the concentration of the drugs required to reduce the maximal effect of the agonist by 50%. This has produced a series of standardised values in brain tissue, that characterise the receptor type, and can be employed to identify other 5HT binding sites in various tissues, since these values are constant and independent of the tissue type. The terminology of serotonin-S1 or 5HT1 binding sites was introduced by Peroutka and Snyder in 1979, and are preferentially labelled by [^3H]-5HT and [^3H]-LSD. They bind 5HT at nanomolar concentrations, exhibiting a K_d in the nanomolar range. Type 1 receptors have higher affinities for indole agonists, compared with type 2 receptors (described below), and lower affinities (in the

micromolar range) for classical 5HT antagonists. In recent studies employing labelled ligands, neuroleptics, β -blocking agents and antidepressants, Nelson (Nelson et al., 1980a), proposed that type 1 5HT sites labelled with [3 H]-5HT were in fact an average measure of [3 H]-5HT to a series of type 1 receptors. He proposed that 5HT_{1A} binding sites exist, which bind spiroperidol and [3 H]-5-HT. Hoyer in 1985, further proposed the existence of three pharmacologically different 5HT₁ recognition sites: 5HT_{1A} which show high affinity for 8OHDPAT and spiroperidol; 5HT_{1B} which shows high affinity for RU 24969 and β -blockers; 5HT_{1C} which is also labelled with [3 H]-mesulergine and is particularly concentrated in the choroid plexus of the rat and pig brain. In the latter case, these receptors are closely analogous to type 2 receptors in that they too, are labelled with [3 H]-mesulergine, but 5HT has a higher affinity for type 1 receptors, and type 2 receptors are differentially labelled with [3 H]-ketanserin. In general, type 1 receptors appear to predominate in all brain regions with the exception of the cerebral cortex where the same numbers of type 1 and 2 receptors are found. Type 2 receptors are labelled with [125 I]-LSD and [3 H]-spiperone. (Peroutka and Snyder et al., 1979), but with the arrival of [3 H]-ketanserin, more specific labelling and binding to 5HT₂ receptors has become possible (Leysen et al. 1981). Further, all known 5HT antagonists (cyproheptadine, cinanserin, mianserin, ketanserin, methysergide, metergoline...) bind with nanomolar concentration to these receptor sites, whereas the 5HT agonists bind with micromolar

affinity, and other neurotransmitters such as dopamine, gamma-amino butyric acid GABA, noradrenaline, histamine and acetylcholine, virtually do not bind to this site. The highest levels of type 2 receptors are localised in the mammalian cerebral cortex and caudate. Various behavioral models using small animals, have indicated that the binding affinities for the compounds used in displacing ketanserin and other type 2 agonists from 5HT₂ receptors, are in close agreement with the values obtained in inhibiting tryptamine and 5HT induced clonic seizures of the forepaws in rats, or mesulergine induced head twitching in rats (Niemegeers et al., 1983). Type 2 receptors are thought to mediate the centrally localised functions. It is important to mention that receptor binding studies should correlate with physiological observations as seen above; further, a change in receptor number and/or sensitivity would concede a physiological role and in this instance, administration of antidepressants in rats carried out by Peroutka et al., 1980, indicated a reduction in 5HT₂ receptor binding as well as reduced density of sites. Chronic treatment of rats with tricyclic antidepressants such as amitriptyline, which inhibits 5HT and noradrenaline reuptake into nerve terminals, was found to reduce 5HT₂ receptor sites, but this down regulation follows a slow time course (2 - 3 weeks), which may match the delayed onset of the therapeutic efficacy of the drug in depressed patients. This has led to a tentative hypothesis for increased supersensitivity of 5HT₂ receptors in depressed patients that may in part be responsible for their

symptoms. Conversely, lesioning of 5HT neurones and depletion of 5HT content, for example by intracerebral administration of the toxin 5,7 dihydroxytryptamine into rats, produced supersensitivity and increased binding of [3 H]-5HT in rat brain membranes when 5HT levels were reduced (Nelson et al., 1978). This was related to an increase in the density of these receptors, thus a form of up regulation (as seen for AII receptors and ACTH receptors) appears to exist for 5HT receptors.

The effects of 5HT in the vasculature are complex; they include venular constriction, arteriolar dilatation, venous and arterial contraction of smooth muscle and thus the net effect in a given vasculature is determined by the balance between vasodilatation and vasoconstriction. In addition, 5HT has been shown to amplify the responses to other stimuli in the vasculature (see below).

In vitro studies have demonstrated that there are marked differences in sensitivity in various vascular beds to 5HT, both within the same species and in differing species. As an example, Van Neuten et al., 1981, showed that the ED₅₀ values for 5HT in several canine vessels, ranged from 0.3nM in the basilar artery to 960nM in the gastrosplenic vein and in the rat aorta the ED₅₀ was 6000nM, however the ED₅₀ alone does not characterise the receptor type but rather the concentrations of antagonists and agonists required to displace the binding of labelled ligand, which under ideal conditions should correlate with the affinity constants of drug-induced physiological effects.

Serotonin induces contraction in several vascular beds including canine basilar arteries, rabbit aorta and femoral artery, rat caudal artery, cat tracheal and bronchial smooth muscle, and the perfused hindquarter of the rat (Cheng et al., 1980., Peroutka et al., 1982, Cohen et al., 1981, Stollak et al., 1982., Van Neuten et al 1981, 1982a, 1982b., Chand 1981., Black et al 1981., Leysen 1982). In most vessels the constrictor effects can be antagonised by 5HT antagonists, which cause a parallel shift to the right in the dose response to 5HT, without affecting the maximal response; the antagonist ketanserin, has been shown to specifically block the contractile and vasodilatory effects of 5HT in a number of vascular beds despite differing sensitivities to 5HT (VanNeuten et al 1982a, 1982b., 1983), in for example the rat caudal artery, and the arteries and veins of the dog and rabbit. Ketanserin blocks the effects at lower doses compared to methysergide, as seen in canine vasculature comparing the two drugs (Van Neuten 1981). Methysergide also has undesirable agonistic properties. Van Neuten showed that type 2 receptors exist in the rat caudal artery and gastrosplenic artery of the dog whereas in the canine basilar and carotid artery the effects of ketanserin antagonism were not as clear cut (Peroutka et al, 1982).

In vascular tissue, it is generally agreed that 5HT interacts with type 2 receptors. In addition Van Neuten et al., 1981, observed that in rat caudal artery, 5HT potentiates the response to low doses of norepinephrine induced contraction and that this could be

inhibited by ketanserin at low doses, indicating that type 2 receptors are responsible for the amplification effect; in the rabbit femoral artery, 5HT amplifies the effects to other noradrenergic agents including AII, histamine, and prostaglandin $F_{2\alpha}$ (Van Neuten et al 1982a) and this amplification effect was specifically inhibited by low doses of ketanserin and not affected by methysergide. Ketanserin inhibited the response at similar concentrations, regardless of the agonist used, which suggests that type 2 receptors are involved; in contrast, the rabbit ear artery constricts on treatment with low doses of 5HT and norepinephrine, but the effect is blocked by phentolamine, an α -antagonist, indicating an interaction of 5HT with α -receptors in some vasculature beds, including canine saphenous vessels (Black et al., 1981.)

In regard to 5HTs interaction with α -receptors, Fozard (Fozard et al., 1983b), has argued that the antihypertensive effects of ketanserin are due to its interaction with α_1 receptors, however even at low intravenous doses of 10mg/ml Wenting (Wenting et al., 1984), and others were able to show an attenuation of high blood pressure with patients on ketanserin treatment and with efferent sympathetic lesions, that excluded the α -components of vasoconstriction. It is conceivable that 5HT interacts with both type 2 receptors and α -receptors to augment the hypertensive state, but may also be species dependent.

Other types of 5HT receptors that do not fit neatly into an identified receptor type include the pre-synaptic 5HT receptor

effects, which include an inhibition of norepinephrine release from postganglionic noradrenergic nerves. This pre-junctional effect of 5HT is different from the receptor types classified, but may also be present in rabbit basilar artery and rat vas deferens (Hay et al., 1982). Other types of serotonergic interactions with tryptaminergic specific receptors, have been characterised in brain tissue using [³H]- tryptamine as ligand (Cascio and Kellar 1982), which allow 5HT stimulated effects; similarly in the rat fundus, tryptamine and a number of other indoleamines, have been shown to exert their effects through serotonergic receptors overlapping α -receptor types, e.g. the sensitivity to tryptamine can be maintained in the rat fundus even when the response to 5HT is blocked by phenoxybenzamine, a non-competitive antagonist (Cohen et al., 1985); other reported indoleamine interactions with possible physiological relevance are N-Acetyl 5HT which interacts with 5HT receptors. This amine can also be detected in large amounts in brain tissue (Niles et al., 1983). Other indoleamines that show high potency in the vasculature such as 5-methoxytryptamine, and also in adrenal steroidogenesis (Muller 1970., Al-Dujaili 1982), may also interact with uncharacterised 5HT receptors, or with type 1 or 2 receptors; however, the field remains unexplored as yet.

5.5 5HT RECEPTOR MECHANISMS OF STIMULATION: ADRENAL AND OTHER TISSUES

In relation to 5HT stimulated steroidogenesis, 5HT receptor sites in the adrenal have so far, not been extensively investigated. Albano

et al., 1974 showed however, that at high doses of 5HT, i.e. 10^{-4} M, an increase in cAMP was detected. Similarly, Tait et al., in 1975, noted an increase in cAMP with higher doses of 5HT, although the steroid output was appreciable at the lower doses of 5HT (10^{-8} M) when cAMP was not significantly elevated. A noteworthy observation made by the Tait (1975), was that incubating cells with a high dose of exogenous cAMP and adding increasing doses of 5HT (10^{-9} M - 10^{-4} M), caused potentiation of the steroid response at 10^{-9} M and appeared to be maximal at all other doses with cAMP present. In this respect, lower doses of 5HT may stimulate steroidogenesis without the involvement of cAMP. Over a dose range of 10^{-8} M - 10^{-5} M 5HT, Fujita (Fujita et al., 1979) also observed an increase in cAMP levels. Further, Williams 1981 noted in studying Ca^{2+} efflux in zona glomerulosa cells stimulated by ACTH, AII, potassium and 5HT, that 5HT did not cause Ca^{2+} efflux in contrast to AII, which suggested that 5HT mediated steroidogenesis did not markedly elicit steroid secretion by mobilising intracellular $[\text{Ca}^{2+}]$. Fakunding (1979) was able to demonstrate in isolated rat and dog zona glomerulosa cells, that altering extracellular Ca^{2+} concentrations affected the maximal response to AII, K^{+} and 5HT-stimulated steroidogenesis without affecting the ED_{50} . Further, Ganguly (1985) also demonstrated an inhibition of the steroid response to 5HT with verapamil, again indicating an important role for Ca^{2+} . It may be that Ca^{2+} in this case is specifically required for the initial calmodulin-dependent phase of the response, since it is already known that both ACTH- and

K⁺- induced steroidogenesis includes this Ca²⁺ dependent phase as well as cAMP in the sustained steroid response phase (Kojima 1985). Thus, 5HT may also follow this mode of stimulating steroidogenesis although there may also be a clear dissociation between the two modes and could be interpreted to suggest several receptor types. The response could be linked to a heterogenous group of 5HT receptors present in the adrenal cortex some of which are cAMP linked and/or Ca²⁺ linked.

The mechanisms of stimulation through different 5HT receptors has been investigated in several species and most commonly in brain tissue e.g. in rat hippocampus the 5HT_{1A} receptor has been linked to cAMP production with a rank order potency of

5-carboxamidotryptamine > 8OH-DPAT = 5HT (Hoyer 1985). Fillion (1978) obtained 5HT stimulation of brain adenylate cyclase in the nM range; other evidence has suggested that 5HT receptors are linked to guanine nucleotides which often reflects an association with adenylate cyclase since GTP is capable of binding to two distinct proteins, N_s which stimulates adenylate cyclase, and N_i that inhibits the enzyme. Guanine nucleotides decrease the binding of [³H]-5HT to 5HT₁ receptor sites but not the binding of [³H]-spiroperidol to type 2 receptors. However as often is the case, the results are not clear cut and Titeler (1984) showed that GTP reduced the binding of 5HT agonists to [³H]-ketanserin type 2 receptor sites and postulated a modulatory role for GTP which was linked to adenylate cyclase activation.

Nelson (1980) was able to demonstrate in rat brain tissue, that 5HT receptors that bound [³H]-5HT could either be linked to adenylate cyclase or be completely dissociated from the complex. Enjalbert et al., 1978 also found adenylate cyclase linked 5HT receptors in brain regions of new born rats with a $K_a = 1\mu M$ for cAMP production. However, 5HT₁ receptors that are linked to adenylate cyclase have not been associated with any clear physiological role except perhaps in Peroutka's study in which 5HT₁ receptors identified by ligand binding studies, were associated with canine vasculature vasoconstrictions (Peroutka et al., 1982); here the cAMP levels were not looked at so it is not known whether this bears any relevance as a second messenger.

Type 5HT₂ receptors have been linked to a Ca^{2+} -PI role. For example, in platelets prelabelled with ³² P, stimulation of aggregation with 5HT produced a parallel increase in ³² P incorporation into phosphatidic acid formation. The K_d for 5HT binding to the 5HT₂ receptor was found to be $3 \times 10^{-7} M$ comparable to a K_i of phosphatidic acid formation of $3 \times 10^{-7} M$; the latter value was determined by comparing the order of potency of 5HT antagonists in inhibiting phosphatidic acid formation, to the potency of antagonists in inhibiting [³H]-ketanserin binding to type 2 receptors in rat brain prefrontal cortex tissue (de Courcelles et al., 1985). A similar coupling of PI hydrolysis to type 2 receptors, has been described in primary cultured embryonic rat frontal cortical cells (Geaney et al., 1984). While it is tempting to assume that these

receptors are directly linked to PI hydrolysis, it must be borne in mind that in platelets, ADP, epinephrine and the ionophore A23187, all stimulate PI hydrolysis, and can all be blocked with a cyclooxygenase inhibitor. These agents stimulate phospholipase A₂ activity and arachidonic production. The metabolites of arachidonic acid stimulate PI turnover as diagrammed (Fig.5.2) so it is possible that 5HT's stimulation of PI is an indirect effect of arachidonic acid metabolism. However, Conn et al., 's study 1986 in rat cerebral cortex showed that 5HT stimulated PI turnover was not blocked by inclusion of cyclooxygenase inhibitors such as indomethacin, or lipoxygenase inhibitors such as BW755C, and followed a pharmacology of PI hydrolysis similar to that in the rat aorta (Roth et al., 1984). Conn concluded that 5HT could directly stimulate receptor mediated phosphoinositide hydrolysis. Indeed, diacylglycerol, a product of PI turnover is rich in arachidonic acid and could provide a suitable substrate for leukotriene production rather than through a direct result of phospholipase A₂ activation, since it is not fully established whether this enzyme is directly activated by hormone interaction or indirectly through phospholipase C activation (Rasmussen and Barrett 1984). In adrenal zona glomerulosa cells Farese (1981,1983) looked in particular at the effects of K⁺, AII and ACTH on PI turnover and in the limited studies with 5HT, they observed that 5HT, like AII and K⁺, specifically increased the hydrolysis of phosphatidylinositol, which paralleled the increase in steroid output. These observations indicate a possible cAMP

independent role of receptor occupancy or one in which Ca^{2+} -PI mediated response are coupled to a cAMP response as indicated above. However, they do not prove that the PI hydrolysis per se is a prerequisite for steroid production. To date, the only direct linkage between PI hydrolysis and Ca^{2+} induced secretions for 5HT, has been seen in the salivary gland of the blow-fly (Berridge 1979). In experiments that involved reducing the PI content of the plasma membrane, the reduction in PI hydrolysis was associated with a reduced stimulation of salivary secretions.

Prostacylin, a product of arachidonic acid metabolism via the cyclooxygenase pathway (see Fig.5.1) has also been linked to type 2 receptor stimulation. Coughlin (1984) looked at cultured bovine aortic smooth muscle cells, and showed doses of 10^{-7} M 5HT- 10^{-5} M 5HT caused an increase in prostacylin production in a dose dependent way which was Ca^{2+} dependent. This would be the case in which phospholipid breakdown requires Ca^{2+} dependent phospholipase A_2 activity to produce arachidonic acid, the precursor of prostacylin. That similar receptors may exert their stimulatory effects on steroidogenesis is possible given that Ellis (1978) observed that cat adrenocortical cells could be stimulated to secrete steroid by increasing doses of prostacylin. ACTH is known to stimulate prostacylin production, so that a possible interaction between cAMP and prostacylin could exist. Prostacylin particularly at higher doses (10^{-6} M), can also increase cAMP levels, so whether it may act as second messenger and cAMP as a third messenger in ACTH and indeed

in 5HT stimulated steroidogenesis, is speculative.

5HT receptors linked to Ca^{2+} channels have been described in the neurones of the species, Aplysia (Corrent et al., 1978., Pellmar et al., 1979). Serotonin induces a Ca^{2+} current which allows for neurotransmitter release at the post-synaptic nerve terminals. In other tissues where pre-synaptic 5HT receptors have been described (as above), it is possible that 5HT is closely associated with a Ca^{2+} channel, in this regard PI hydrolysis may control Ca^{2+} gating as Michell(1977) has hypothesised. In the vas deferens, 5HT induces both phasic and rhythmic contractions of the vessel walls. The phasic component can be completely abolished by methysergide whereas the rhythmic component is blocked by Ca^{2+} channel blockers including nifedipine and verapamil, which might be associated with 5HT- Ca^{2+} channel interactions (Hay et al., 1982).

In adrenocortical cells, Ganguly (1985) showed that verapamil, a slow Ca^{2+} channel blocker, inhibited 5HT stimulated steroidogenesis but whether the effects are due to a Ca^{2+} -PI linked receptor or Ca^{2+} channel per se with concomittant activation of Ca^{2+} influx, is not clear. However the overall conclusions appear to be that 5HT mediated steroidogenesis requires Ca^{2+} at some point beyond the initial binding site interaction, in order to modulate the magnitude of the response. Caution must also be made in using Ca^{2+} blockers in non-excitabile tissues, as Rubin (1982) has indicated that the effects may be a result of local anaesthetic effects on the cell membrane and Ca^{2+} channel blockers in adrenal zona glomerulosa cells

have been shown to inhibit 5HT stimulated steroidogenesis.

From the above evidence it would appear that the receptor-coupled mechanisms for 5HT are not clearly discernible. Indeed it is debatable that adenylate cyclase in the brain and possibly in the adrenal, at the lower concentrations of 5HT which stimulate steroidogenesis without affecting cAMP, has physiological relevance in the coupling response. This will be discussed more fully in the following chapters.

Chapter 6

METHODOLOGY

6.1.1 Preparation of Isolated Zona Glomerulosa Cells

In all in vitro experiments female Wistar rats were used.

Adult female rats of 180-200g were taken from cages containing a maximum of six rats per cage, and killed between 9.00a.m.-10.30a.m. by cervical dislocation. On average, eight rats were used for steroid experiments. An incision in the abdominal midline was made and the adrenal glands were quickly removed with surrounding fat and put into saline flasks placed on ice until digestion.

6.1.2 Collagenase Digestion

After trimming of adhering fat, each adrenal was placed in a petri dish with Whatman 5 hardened filter paper settled on the base of the dish, and soaked in ice cold saline. The adrenal was held firmly by a small pair of curved stainless steel forceps and partially cut along the middle with a sharp scalpel blade, until two hemispheres attached by a piece of uncut connective tissue was obtained. Using the back of the scalpel blade and holding the hemispheres cut-face downwards, the inner cortex and medulla were gently pushed out leaving the capsule and attached zona glomerulosa cells for further processing (Haning et al., 1970).

All capsules were placed in ice cold saline until all the adrenals had been dissected and then, using a fine pair of forceps, all capsules were placed in a pre-warmed solution of collagenase

(Worthington) dissolved in 20g/l bovine serum albumin (2% BSA) Krebs Ringer Bicarbonate solution pH7.4.

The composition of the Krebs-Ringer solution is shown Table 6.1:

Table 6.1

Molarity		Volume (mls)
0.77M	NaCl	81.4
0.77M	KCl	1.76
0.55M	CaCl ₂	2.4
0.77M	KH ₂ PO ₄	0.8
0.77M	MgSO ₄	0.8
0.154M	NaHCO ₃	84.0

The solution was made up to a final volume of 520 mls with double distilled water (DDW); other total volumes were obtained by scaling the basic recipe.

The solution was gassed in a mixture of 95%CO₂ /5%O₂ for 30 minutes and glucose (BDH Chemicals, Poole, Dorset.) at a final concentration of 0.2% was gently dissolved into the solution. For the digestion step BSA (Fraction V, Miles Scientific, U.K.) was added at a final concentration of 2% to Krebs and pre-warmed in a water bath at 37⁰C. Crude collagenase at a final concentration of 2 mg/ml was added to Krebs solution (purified forms have little effect on tissue digestion).

The whole capsules were placed in Krebs 2% BSA (0.2% glucose) and left in a shaking water bath 120Hz at 37°C for thirty minutes. After this time the digestion mix was aspirated thirty times, using a 5 ml pipette with the tip cut to a diameter of approximately 1mm, to allow free dispersion of aspirate up and down the tip length. The cell suspension was then allowed to digest a further thirty minutes and resuspended again for a further thirty strokes. The cell suspension at this stage appeared homogeneous and was gently filtered through a 100µm nylon gauze attached to the cut end of a 5ml plastic syringe. The cell suspension was filtered into equal volumes into 10ml plastic conical centrifuge tubes and after balancing, placed in the MSE coolspin centrifuge (Mistral model 4L) for 20 minutes at 400g and 4°C. This procedure was repeated three times with two washes of the cell pellets between spinning in Krebs-Ringer buffer (2% BSA), and after the final spin the clear supernatant was discarded and the cell pellet resuspended in 5mls Earle's Medium M199 (M199 Flow Laboratories) without sodium bicarbonate (0.2% BSA, 0.2% Glucose) modified to a concentration of 3.6mmol K⁺/l to give the same ionic concentration as the Krebs solution. The reason for using M199 was because experiments carried out in Krebs buffer (0.2% BSA) showed poor aldosterone responses when AII was used as the stimulus. These observations have also been noted by other workers e.g. Campbell et al., (1982) showed that M199 increased both the basal and stimulated levels of aldosterone. Although in my experiments (and as reported by Tait et al., 1972, 1980a and 1980b) it was found that AII only

stimulated steroidogenesis in Krebs incubates containing 4% BSA, we did not continue with this regime because of the high cost of the albumin used for all the experiments; the high albumin concentration also caused loss of sensitivity in the direct steroid radioimmunoassays employed. The recipe used for reconstituting the M199 used in the studies was as follows.

Medium 199 powder was dissolved in 100 mls of DDW and dispensed as aliquots into 10ml tubes. A working buffer solution was prepared with the following salts all of which were of Analar grade from BDH chemicals Ltd., Poole, Dorset, U.K. The ionic composition of M199 is shown in Table 6:2.

To this was added 32.36 mls of DDW to make a working solution of 75mls (3.6mM K^+). Further increases in working volumes were made with the appropriate scaling. For experiments where different K^+ concentrations were required the volume of NaCl was adjusted such that the total volume of (NaCl + KCl) was 20 mls as shown in Table 6:3.

Table 6:2

Molarity		volume(mls)	
0.154M	NaCl	20.00	
0.154M	KH ₂ PO ₄	0.11	
0.154M	Na ₂ HPO ₄	0.135	
0.154M	MgSO ₄	0.32	
0.154	NaHCO ₃	12.10	
0.11	CaCl ₂	0.88	
	M199	5.00	

Table 6:3

Final K ⁺ [mM]	NaCl(mls)	KCl(mls)
3.60	20 00	---
5.00	18.16	1.83
8.40	16.92	3.08
13.00	15.24	4.76
15.00	14.5	5.50

6.1.3 Counting Cells in the Haemocytometer

To a 100 μ l aliquot of cells a 1:10 dilution of 4% Trypan blue was added i.e. 1ml and left to stand for 2 minutes to allow diffusion through the cells.

A small amount of this suspension was placed at the edge of the cover slip adhering to an improved Neubauer haemocytometer, and allowed to spread by capillary action, across the counting chamber. The haemocytometer was viewed with a Nikon model type 104 optical microscope and glomerulosa cells excluding Trypan blue (i.e. viable cells) were counted. All cells within the four outside squares and the middle finely divided square were counted and the number of cells, multiplied up by the correction factor to a total volume of 1ml. This number was then multiplied by the total volume of the original cell suspension i.e.

$$\text{NUMBER OF CELLS} = \text{NUMBER IN COUNTS} \times 2000 \times 5\text{MLS (TOTAL VOLUME)}$$

6.1.4 Incubation of Cells

In all studies zona fasciculata/reticularis cells accounted for less than ten percent contamination as previously seen by Haning (1970). The 5ml cell suspension was placed in a plastic beaker and allowed to incubate in a shaking water bath at 37°C and 160 rpm for a total of thirty minutes. After this preincubation time the cell suspension was thoroughly mixed by swirling and several aspirations, using a 5ml plastic pipette, before being transferred to 10ml conical centrifuge

tubes in equally distributed volumes and spun down for a further 10 minutes at 300g and 4⁰C. The resultant supernatant was carefully aspirated off and the cell pellet gently resuspended in fresh M199 (0.2 %BSA and 0.2% Glucose) to a total volume that gave 100,000 cells per 400µl aliquot. In all experiments an ice tray containing water/ice mixture was used to cool the incubation tubes prior to setting up the static incubates. All solutions and cell suspensions were also kept in the ice tray whilst taking aliquots. Initially a 500µl aliquot of M199 (0.2% BSA) was pipetted into all incubation tubes followed by 400µl of cells. All tubes were given a gentle shake and the incubates were then made up to a final volume of 1ml by the addition of 100µl of buffer/stimulus. In the case of drug antagonism studies, 100µl of antagonist was added to the 400µl of cells with 400µl of buffer, and pre-incubated for 15 minutes before further addition of 100µl of buffer/stimulus. All tubes were again gently shaken to allow complete dispersal and distribution of stimulus to the cell suspension. All solutions of the stimuli were made up in M199 (0.2% BSA), to a concentration 10 times greater than that required in the final incubation, so that 100 µl added to a total volume of 1ml gave the required concentration. The incubation tubes were transferred from the ice bath to a shaking water bath operating at 100 ~~cymin~~ ¹ at 37⁰C and incubated for thirty minutes under a ventilation hood with 95% CO₂/5% O₂.

The incubation tubes were then removed from the water bath and placed directly on ice for 10mins to inhibit further steroid biosynthesis

before being transferred to pre-chilled centrifuge carriers at 4°C. All tubes were centrifuged at 4°C in the MSE centrifuge at 800g for 20 minutes. The resultant supernatant was carefully decanted into clean test tubes (LP4), and frozen at -20°C until assayed for steroid hormones.

Preliminary experiments using 5HT as stimulus, showed that steroid output was linear with time for incubation times of 30 minutes to 2 hours.

6.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC was used to measure 5HT and its metabolites since it is one of the most precise methods for determining biogenic amines in biological fluids and tissues (Sasa et al., 1977, Mefford 1981, Achilli et al., 1983).

This method is sensitive to concentrations as low as 10-20pg/injection. The intra-assay coefficient of variation for the system described in this thesis has been determined as 2.1% ± 3.1% and the inter-assay coefficient of variation was 5.7% ± 8.2 according to Gow et al., 1987.

6.2.1 Reagents

5-hydroxytryptamine creatinine sulphate and 5-hydroxyindole acetic acid were obtained from Sigma (Dorset, Poole.) and internal standard N-ω-methyltryptamine from Aldrich Chemicals. HPLC grade methanol and water for the mobile phase was purchased from Rathburn Chemicals Ltd., Walkerburn, Peebleshire, Scotland. Reagent grade sodium

dihydrogen orthophosphate (NaH_2PO_4) was obtained from BDH. The mobile phase consisted of a 3mM NaH_2PO_4 buffer containing 15-17.5% methanol, depending on resolution and retention time requirements of the amines studied. The disodium salt of ethylene diamine tetraacetic acid (EDTA 1mM) was added to the mobile phase and purchased from BDH. Pairing ion agent (octane-1-sulphonic acid sodium salt) was from HPLC Technology Ltd.

The final concentration of salts and solvents in 1 litre of the mobile phase are given in Table-6:4.

Table 6:4

WATER	850mls
METHANOL	150mls
EDTA	400mg
PIA	120mg
NaH_2PO_4	5.4g

The mobile phase was filtered and degassed by passing through a 0.2 μm Millipore 'Durapore' filter (Waters, Millipore, Harrow, Middlesex) under reduced pressure. Standard solutions were prepared in a solution (pH 1.8) of 1.5% (0.15M) perchloric acid and 0.2mM cysteine (Sigma) and these were diluted to the desired concentration in the same solvent.

6.2.2 Apparatus

Liquid chromatography was performed using a Waters M600A pump with a Waters U6K injector and 2ml sample loop for injection into the system. A pre-column (3.9mm x 2cm) of C₁₈ Corasil was used (particle diameter 35-50µm) which consisted of C₁₈ groups linked to particles of silica. It's function was to filter out large particles of contaminating matter which would have affected the analytical column, decreasing both it's resolution and it's lifetime. The analytical columns used were (1) 3.9mm x 30cm packed with C₁₈ µ Bondapak (particle diameter 10µm) from Waters Ltd., or (2) a 4.0mm x 10cm column (Capital HPLC, Edinburgh, U.K.) packed with Hypersil ODS (particle diameter 5µm) from Shandon Chemicals, Runcorn, Cheshire. Standard conditions were 1ml/minute flow rate at room temperature. Detection was accomplished using a Model LC-4A electrochemical detector (Bioanalytical Systems, West Lafayette, USA) using a working potential of +0.7V with a 5nA full scale deflection for the oxidation of samples by glassy-carbon electrode. Peaks were plotted using a Waters data module, and quantitation of the amines present in blood and tissue samples were made by comparison of the peak heights of the samples to those given by standard concentrations, at the characteristic retention times for the amines.

6.2.3 Sample Preparation

In general, there are two methods of choice for pretreating samples prior to HPLC analysis (1) extraction of the components of interest

or (2) direct injection following deproteinisation. The latter method has been applied in preparing brain and adrenal homogenates (Blanchard, 1981, Kuriyama et al., 1984, Verhofstadt et al.., 1983) and is by far the more rapid and simpler technique, especially in the handling of small tissue or fluid samples.

6.2.4 Adrenal Sample Preparations

Female, Wistar rats weighing 180-220g were obtained from the Animal Unit at the Western General Hospital, Edinburgh. All animals were fed normal rat chow and water ad libitum and were maintained on a 12:12hour light/dark cycle throughout the studies. The rats were always killed at the same time i.e. 9.30am -10.30am to reduce the variation in measurement of biogenic amines which are known to fluctuate during the day (Albrecht et al., 1956).

Rats were sacrificed by cervical dislocation and the adrenals isolated and surrounding fat removed as described above. In some instances capsules were separated from the inner cortex and medulla and these as well as whole adrenals were assayed, or else frozen in polypropylene microcentrifuge tubes(1.5ml) at -70°C .

6.2.5 Tissue Homogenisation

Tissue was weighed whilst still frozen and the appropriate volume of 1.5% perchloric acid i.e. 100mg:1ml acid, containing $2 \times 10^{-6}\text{M}$ internal standard (final concentration) to measure recovery of amines during the procedure, and 0.2 mM cysteine to prevent oxidation of the amines, was added to the samples. The samples were allowed to stand at 4°C for 15 minutes before being processed in a motor driven

homogeniser (Jencons Scientific Ltd., Bedfordshire) with teflon pestle, for 2 minutes at maximum speed setting 10. The resultant homogenate was spun down in the Microfuge (model 154, Camlab, Cambridgeshire) at 15,000g for 15 minutes and 4°C and the resultant supernatant carefully aspirated and samples of this injected into the HPLC column in 25µl aliquots. Where samples were not immediately assayed, these were frozen at -20°C and usually assayed within a week, to reduce the risk of amine degradation. All tissue preparation steps were carried out on ice. All amine values on the column need to be corrected for the volume of acid the tissue is dissolved in; thus, 100 mg of tissue is routinely digested in 1 ml acid so that µM concentrations need to be converted to moles per µl to multiply up the concentration in x µl of tissue homogenate per x mg and thence per gram wet weight.

6.2.6 Serum Samples

A number of modified precipitation methods for prior removal of protein before loading onto the column, have been used (Semerdjian-Rouquier 1981, Blanchard et al., 1981) and modified versions include the one used below (Gow et al., 1987). Rats were anaesthetised with ether and 100µl of blood collected by cardiac puncture into polypropylene conical microcentrifuge tubes containing 900µls of coagulant.

The coagulant cocktail was a modified mixture from Engbaek et al., 1982 and was prepared as described in table 6:5.

In a total volume of 0.9mls used in sample preparations:

Table 6:5

0.1 Unit Thrombin/0.9 mls =	0.11 Units/ml(original stock)
10 nmol Pargyline/0.9 mls =	11.1 μ M
1 nmol Chlorimipramine/0.9 mls =	1.1 μ M

Thrombin augments platelet aggregation and stimulates the release of 5HT from the platelets thus increasing the concentration of serum 5HT. Pargyline inhibits monoamine oxidase activity and thus, the metabolism of 5HT which may occur in blood collections.

Chlorimipramine, prevents the re-uptake of 5HT into platelets thus providing a more accurate measure of whole blood 5HT.

Samples were left to stand at 4⁰C for at least one hour, after which time the tubes were centrifuged at 10,000g and 4⁰C for 15 minutes.

The resultant clear supernatant was transferred to clean tubes before further processing. In a separate set of tubes was added 50 μ l of 1.5M perchloric acid for the deproteinisation of serum, and 50 μ ls of N- ω -methylserotonin dissolved in 0.15M perchloric acid and added for marking recovery of the endogenous amines (Mais et al., 1981) and finally, 400 μ l of serum was added. The tubes were vortexed and left at 4⁰C for 15 min after which, they were centrifuged for 30 min at 4⁰C and 10,000g. The resultant supernatant was collected and 25 μ l samples injected into the column.

Thus, all values obtained on the column need to be corrected by a factor of x12.5 to correct up for 1:10 dilution of coagulant to blood in the first stages, and in deproteinisation 400 µl of sample in a total volume of 500 µl, i.e. $500/400 \times 10 = 12.5$.

6.3 RADIOIMMUNOASSAY

For all in vitro experiments with adrenocortical cells incubated in M199 (0.2% BSA), corticosterone and aldosterone were measured by direct radioimmunoassay of the medium using [¹²⁵I]-corticosterone and [¹²⁵I]-aldosterone respectively as radiolabels.

6.3.1 Radioimmunoassay of Corticosterone and Aldosterone

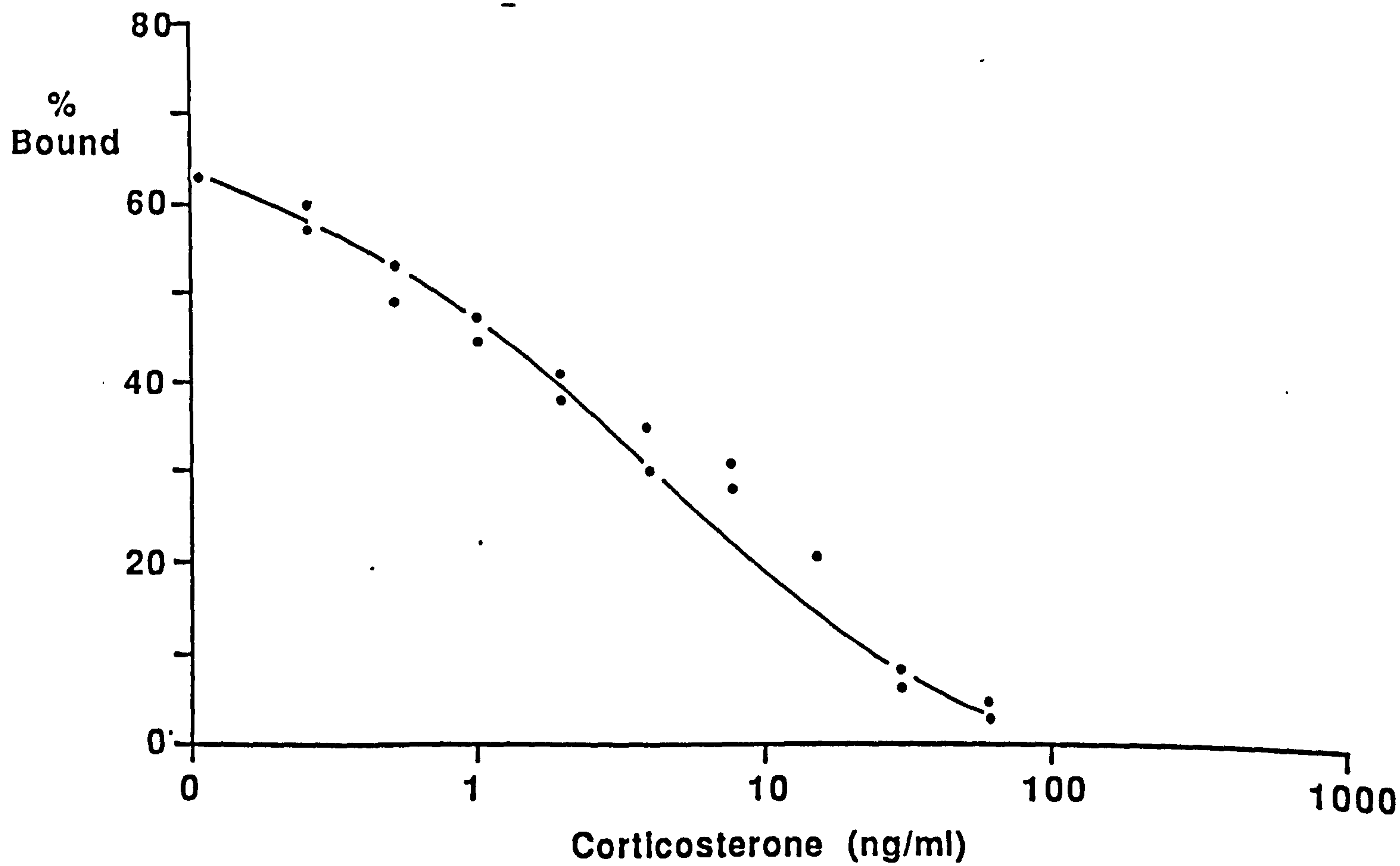
6.3.1.1 Preparation of [¹²⁵I]-corticosterone

This was prepared by the coupling of corticosterone-3-(O-carboxymethyl) oxime to a previously labelled [¹²⁵I]-histamine molecule (Al-Dujaili et al 1981b).

6.3.1.2 Production of antisera

Corticosterone-3-(O-carboxymethyl) oxime was coupled to bovine serum albumin and injected into New Zealand rabbits. The cross reactivities for the resultant antisera has already been described (Al-Dujaili et al., 1981b) and did not exceed 0.03% for 11-Deoxycorticosterone the other major cross-reactant.

Fig 6.1
Typical standard curve to corticosterone in RIA using charcoal
separation method



6.3.1.3 Solutions and Steroid Standards

A stock solution of corticosterone obtained from Sigma was dissolved in absolute ethanol x mg/ml and diluted out in M199 (0.2% BSA) solution with 0.1% azide.

Phosphate buffer 0.05 M pH 7.4 containing 0.1% BSA and 0.1% azide was used as diluent in the steroid assays.

6.3.1.4 Dextran-coated charcoal

This was prepared by making a 2 litre solution as follows:

activated charcoal (Sigma)	12.5g
dextran (Pharmacia Fine chemicals, Upssala, Sweden)	1.25g

both dissolved in 0.1M phosphate buffer and a prewarmed solution of gelatin in phosphate buffer (0.8g) was then added to a total volume of 2 litres. The resultant solution was chilled at 4°C before use in assay separations.

6.3.2 Setting up Incubations

The assay incubates both for aldosterone and corticosterone measurement were such, that the incubation volume contained : 25 µl sample, 200 µl antibody/label mix in 0.05M phosphate buffer

In the corticosterone assay the final antibody concentration was optimum for assay binding at 50%, at 1:60K. The label solution gave counts in the range 3 000-5 000 cpm in the final incubate volume.

The lower level of sensitivity was at 0.5 ng/ml and the working range lay between 0.5-16 ng/ml (see Fig.6.1).

The interassay-coefficient of variation at 0.5 ng/ml was 9.7%, 20.9% at 2 ng/ml, and 21.8% at 16 ng/ml.

Tubes were vortexed and incubated at 4⁰C overnight before charcoal separation. Dextran-coated charcoal kept at 4⁰C was added (volume 0.6 mls) and tubes were centrifuged at 1,720g for 20 min at 4⁰C.

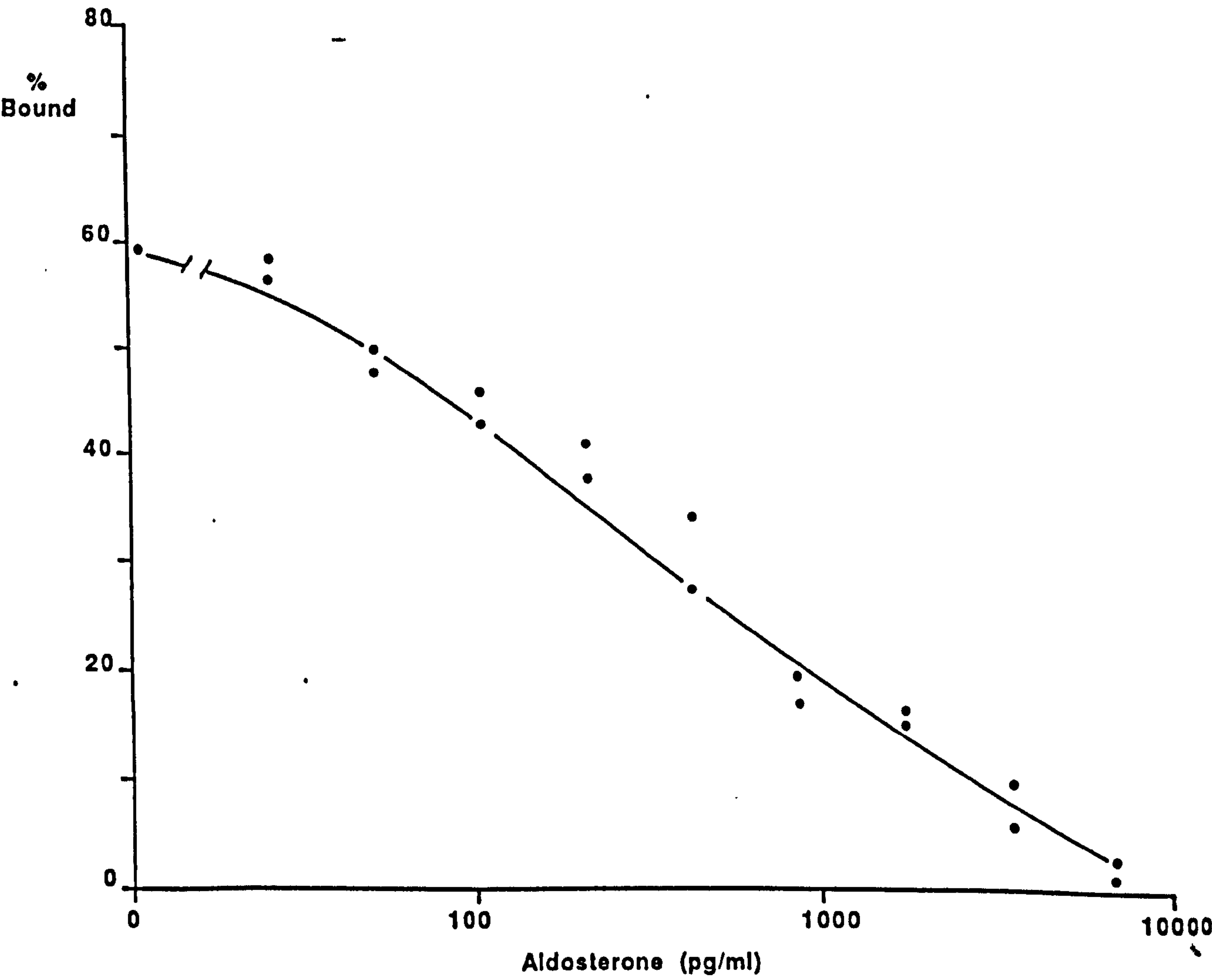
Tubes were aspirated and the charcoal pellet (free fractions), counted in the multiwell gamma counter (model LKB 1260 multigamma).

Aldosterone levels in in vitro samples were measured in a similar direct radioimmunoassay procedure. The aldosterone antibody used was tested for cross reactivity and is tabulated below:-

Table 6:7

<u>STEROID</u>	<u>% cross-reactivity of aldosterone antibody (pH 7.4)</u>
Aldosterone	100
Pregnenolone	<0.00002
Corticosterone	<0.00002
17aOHProgesterone	<0.00002
Testosterone	<0.00002
Androstenedione	<0.00002
Oestradiol	<0.00002
Cholesterol	<0.00002
Cortisol	<0.00002
18OHDOC	=<0.00002
DOC	=<0.00002

Fig 6.2 Typical standard curve to aldosterone in RIA using charcoal separation method



The antibody was used at a final concentration of 1:100K and was a kind gift from Dr.F.A.O. Mendelsohn. The antibody was raised in rabbits and Fig. 6.2 shows a typical standard curve for aldosterone in buffer pH 7.4 (0.2% BSA and 0.1% azide). The lower limit of detection for aldosterone was consistently around 50 pg/ml and the working range for steroid measurement lay between 50-3,200 pg/ml. The interassay-coefficient of variation was 4.4% at 50 pg/ml, 10.3% at 400 pg/ml, and 31.1% at 3,200 pg/ml.

6.3.3. Radioimmunoassay of Plasma Aldosterone and Corticosterone

6.3.3.1 Plasma Aldosterone measurement

Charcoal stripped serum from female Wistar rats was used in preparing aldosterone standards from a stock solution of 1mg/ml. Labelled [¹²⁵I]-aldosterone was prepared by the chloramine-T method as described (Al-Dujaili and Edwards 1981b). Aldosterone antibody (S14-3) was prepared in sheep and used at a final concentration of 1:400K in the incubation samples. The plasma samples were prepared by collection of whole blood into EDTA solution (see collection for PRA assay) and plasma samples were kept on ice before centrifugation. Plasma samples were centrifuged at 3,000g 4⁰C for 20 minutes and the resultant plasma supernatant collected for direct assay or stored frozen at -20⁰C until assay.

A 20 µl sample of plasma or standard was added to polystyrene LP3 tubes in duplicate and aliquots (480 µl) of an antibody/label mix (final dilution of antibody, 1:400K) in phosphate/citrate buffer pH

4.0 (0.1% BSA and 0.1% azide) were added. The tubes were vortexed and incubated overnight at 4⁰C before charcoal separation as described above.

Phosphate/citrate buffer pH 4 was prepared by mixing 15 parts Na₂HPO₄ (0.5M)/citrate buffer(0.25 M) 35 parts as this was found to be the optimum pH for plasma aldosterone measurement (Al-Dujaili et al., 1981c).

6.3.3.2 Radioimmunoassay for plasma corticosterone

Plasma corticosterone was measured by a direct radioimmunoassay of corticosterone (Al-Dujaili et al., 1981b). Blood samples were taken from EDTA treated samples in the measurement of PRA and/or AII and plasma samples collected by high speed centrifugation at 4⁰C as described above. Phosphate/citrate assay buffer (0.05 M, pH 4.0) was used in a total volume of 200 µl together with 50 µl plasma/standard samples. The final antibody dilution was as in the in vitro assay (1:60K) using [¹²⁵I]-labelled corticosterone at 3000cpm.

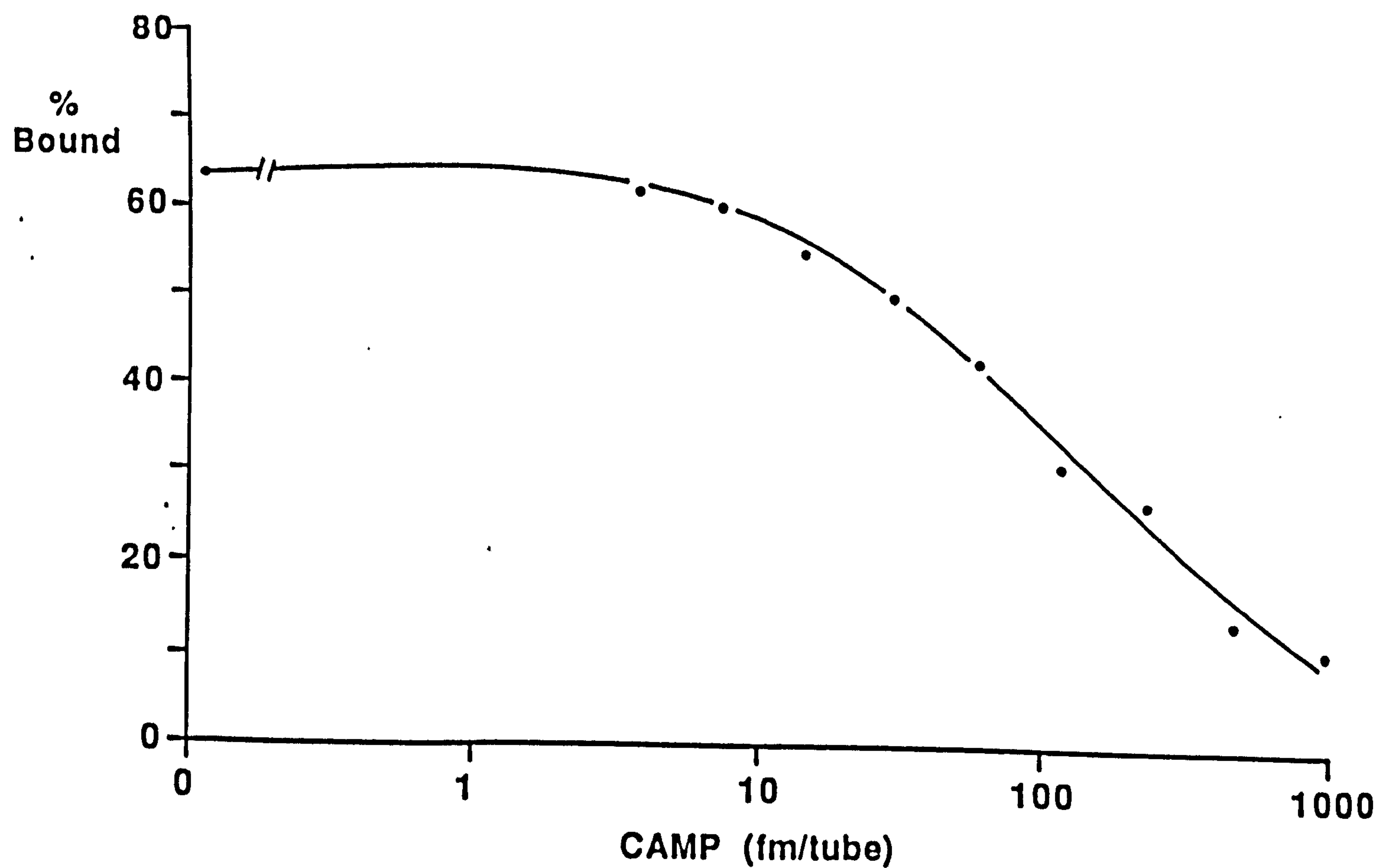
6.4 Radioimmunoassay for cyclic AMP

In measuring cAMP from in vitro samples, an aliquot of 500µl was treated on ice with 5µl of acetic acid (1:4) and vortexed.

Immediately afterwards, the acidified samples were acetylated with 15µl of a freshly prepared mixture of acetic anhydride/triethylamine (1:2). The acetylation reaction was carried out on ice and the vortexed samples were ready for assay.

Fig 6.3

Typical standard curve to cyclic AMP in RIA using charcoal separation method



For radioimmunoassay, the cyclic AMP antibody was raised in rabbits following Harper and Brooker (1975) protocol.

The incubates contained 25µls sample, 250µl citrate/phosphate buffer (pH 5.0) and antibody/label. The final antibody dilution was 1:200K and the inter-assay coefficient of variation was less than 10%. The working range lay between 15-500 fm/tube (see Fig.6.3).

6.5 Assay for Plasma Renin Activity (PRA)

Plasma renin activity is an indirect measure of AII levels in the plasma and is often preferable to the measurement of AII which has a short half life. Generation is achieved by the protocol described below.

6.5.1 Reagents

A 0.1M phosphate buffer solution containing 0.1% azide and 0.1% BSA was used in the assay procedure. An EDTA solution was prepared by dissolving 5g of the disodium salt in 50mls of distilled water at 60°C, and was used as a stock solution for the assay. In the collection of plasma samples 5g of EDTA salt were dissolved in 500ml of sterile saline at 60°C (27 mM). BAL (British Anti-Lewisite - an angiotensin converting enzyme (ACE) inhibitor which prevents AI conversion to AII) was made up in the fume cupboard using 15ml arachis oil + 2,3-dimercapto-propan-1-ol (242 µl) + benzyl benzoate (535 µl) and kept frozen until assays were set up. A solution of 8-hydroxyquinoline (660 mg) was dissolved in 10 mls distilled water and kept at 4°C. Both this compound and EDTA are used to prevent

conversion of AI to AII by by extraneous peptidases present in the plasma.

6.5.2 Treatment of Plasma Samples

Rat blood samples were pipetted into polypropylene conical microcentrifuge tubes on ice, such that a final concentration of 1:10 was made relative to the addition of EDTA (27 mM) Samples were then centrifuged at 4⁰C at 3000g for 60 minutes and the resultant supernatant frozen at -20⁰C until assay.

Generation of AI - Two sets of tubes, one set kept on ice throughout, and another set incubated at 37⁰C were prepared by adding 500 µl to one set of tubes containing a 30 µl mixture of BAL, 8-OH Quinoline and EDTA at 4⁰C. The mixture was vortexed and to this was added 500 µl of 0.1 M phosphate buffer and vortexed again. Out of a total volume of 1.03 mls, 400 µl was pipetted into the other set of empty tubes placed on ice. Both sets of tubes, i.e. 37⁰C and 4⁰C, were incubated for a total time of 120 minutes after which time all tubes were kept on ice.

6.6 RADIOIMMUNOASSAY OF AI GENERATED IN SAMPLES

The assay mixture contained 25 µl (sample/standard) in duplicate, 200 µl *Ab/label mix in phosphate buffer.

The mixture was vortexed, and incubated overnight at 4⁰C and separated by dextran coated charcoal as described above, followed by counting in the multiwell gamma counter. * The antibody (R2B5) was prepared at a final concentration of 1:60K and the label diluted to

give approximately 3000cpm/tube. PRA values were calculated by subtracting counts from tubes maintained on ice from values of AI obtained at 37⁰C. The final value was divided by the time of generation for AI to give values in x ng/ml/hr.

6.6.1 Plasma AII Assay

6.6.1.1 Reagents

The peptidase inhibitor solution was prepared using 4.65g EDTA (chelator of Ca²⁺) in 100ml distilled water with addition of 0.45g o-phenanthroline dissolved in 1ml ethanol. The working assay buffer was prepared by addition of 1.51g TRIS + 250ml water (distilled), and 0.1% BSA with 0.1% azide.

6.6.1.2 Blood Collection

A sample of blood was collected into polypropylene microcentrifuge tubes containing a volume of inhibitor such that the blood:inhibitor ratio was 9:1 and in rat blood collections this was 1.8 mls:0.2 mls inhibitor. The blood sample mixture was collected on ice and centrifuged at 15,000g for 30 mins at 4⁰C. The resultant plasma supernatant was collected and stored frozen at -20⁰C until assay.

6.6.1.3 Preparation for AII Separation from Plasma Samples

Columns of C₁₈ sep-pak cartridges were used in the separation of AII and related peptides from plasma samples. C₁₈ cartridges consist of hydrophobic moieties bound to a column of silica particles and this allows adsorption of peptides and small molecules with the elution of plasma proteins. The benefits of this separation are two-fold in

that it also enables plasma samples of AII to be concentrated. A total volume of 5ml methanol was passed through the columns followed twice by 5ml of distilled water, after which the plasma sample was passed through. Bound peptides were then eluted by passing a final wash of 2.2 mls 80% methanol:20% water over the column. The eluate was collected into glass 12 x 75mm tubes (Corning GlassWorks, Corning, USA) and dried under 95%CO₂:5%O₂ at 35⁰C-40⁰C. When dried 0.5 mls of TRIS buffer was added to each tube and assayed.

6.6.2 Assay Procedure

50 µl of sample/standard was added to 200 µl* Antibody/label mix (* Antibody R6B4 1:300K final dilution and 3000 cpm per incubate). The standard curve range lay between 0.02-10.24 ng/ml (double dilutions).

The assay incubates were left overnight at 4⁰C after which samples were separated by charcoal separation as above and the pellets were counted using a multiwell gamma counter for [¹²⁵I]-AII measurement.

Chapter 7

THE EFFECTS OF 5HT ANTAGONISTS ON THE STEROID RESPONSE IN THE ZONA GLOMERULOSA

The purpose of these studies was to attempt to determine more fully the direct effects of 5HT antagonists on 5HT stimulated steroid output, in particular aldosterone, in the rat zona glomerulosa cells incubated in vitro.

In initial experiments the dose response to 5HT in isolated zona glomerulosa cells was assessed by measuring the output of cAMP, corticosterone and aldosterone over a thirty minute incubation period. In the first instance, only two 5HT antagonists were tested for their antiserotonergic properties in the adrenal.

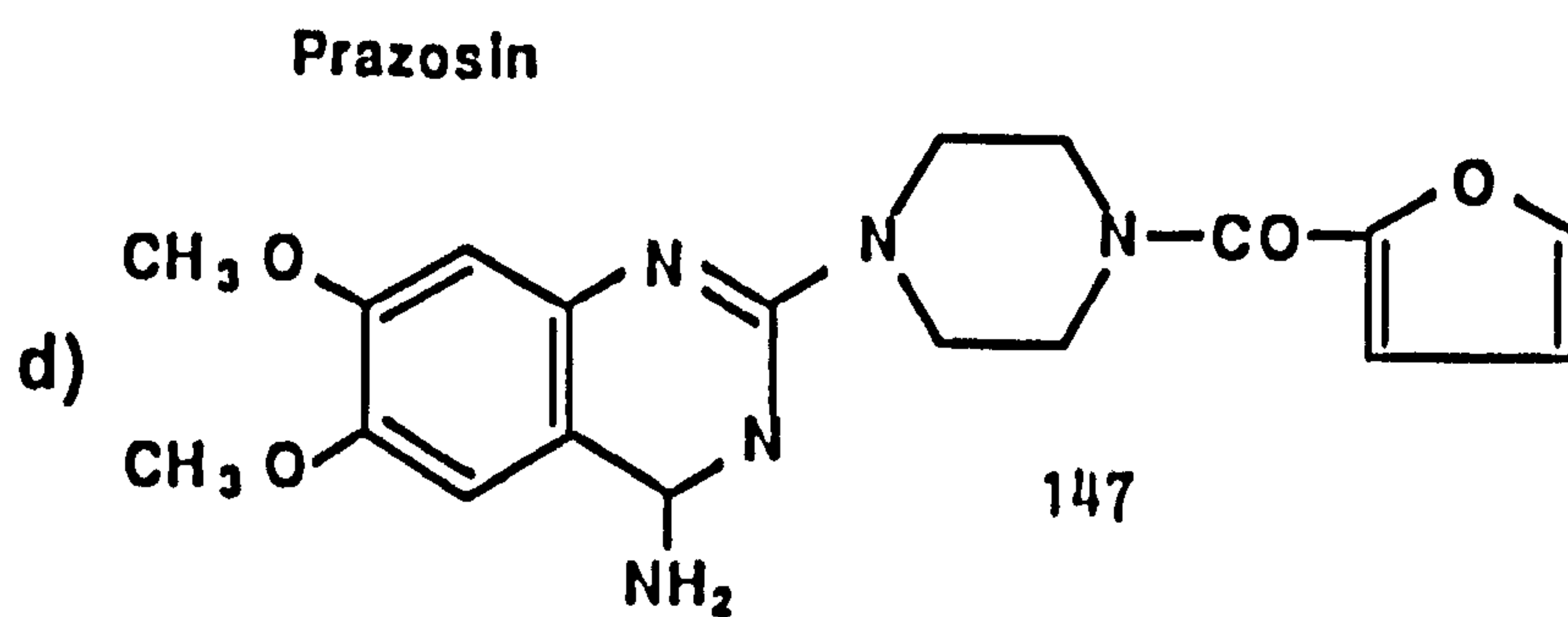
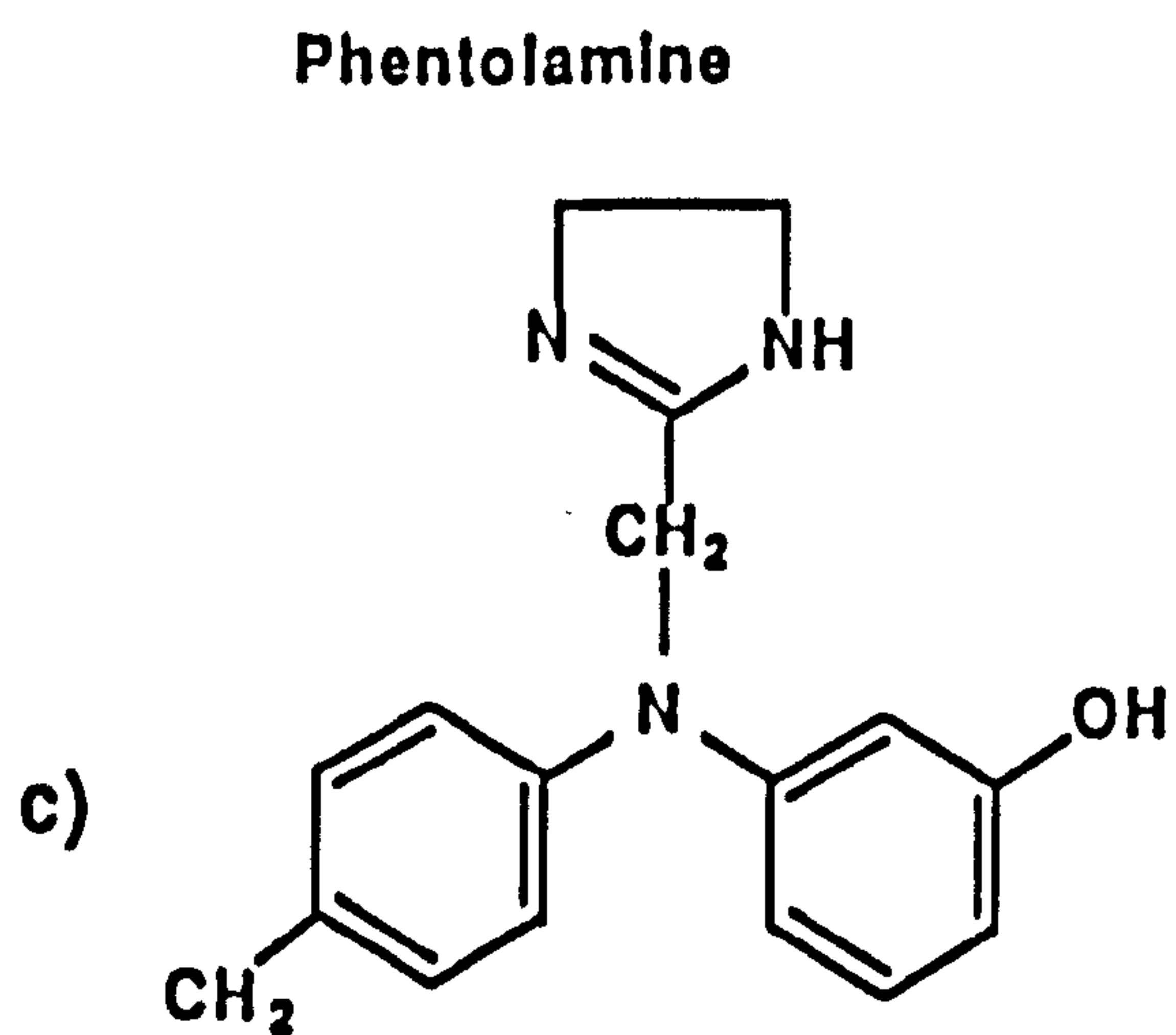
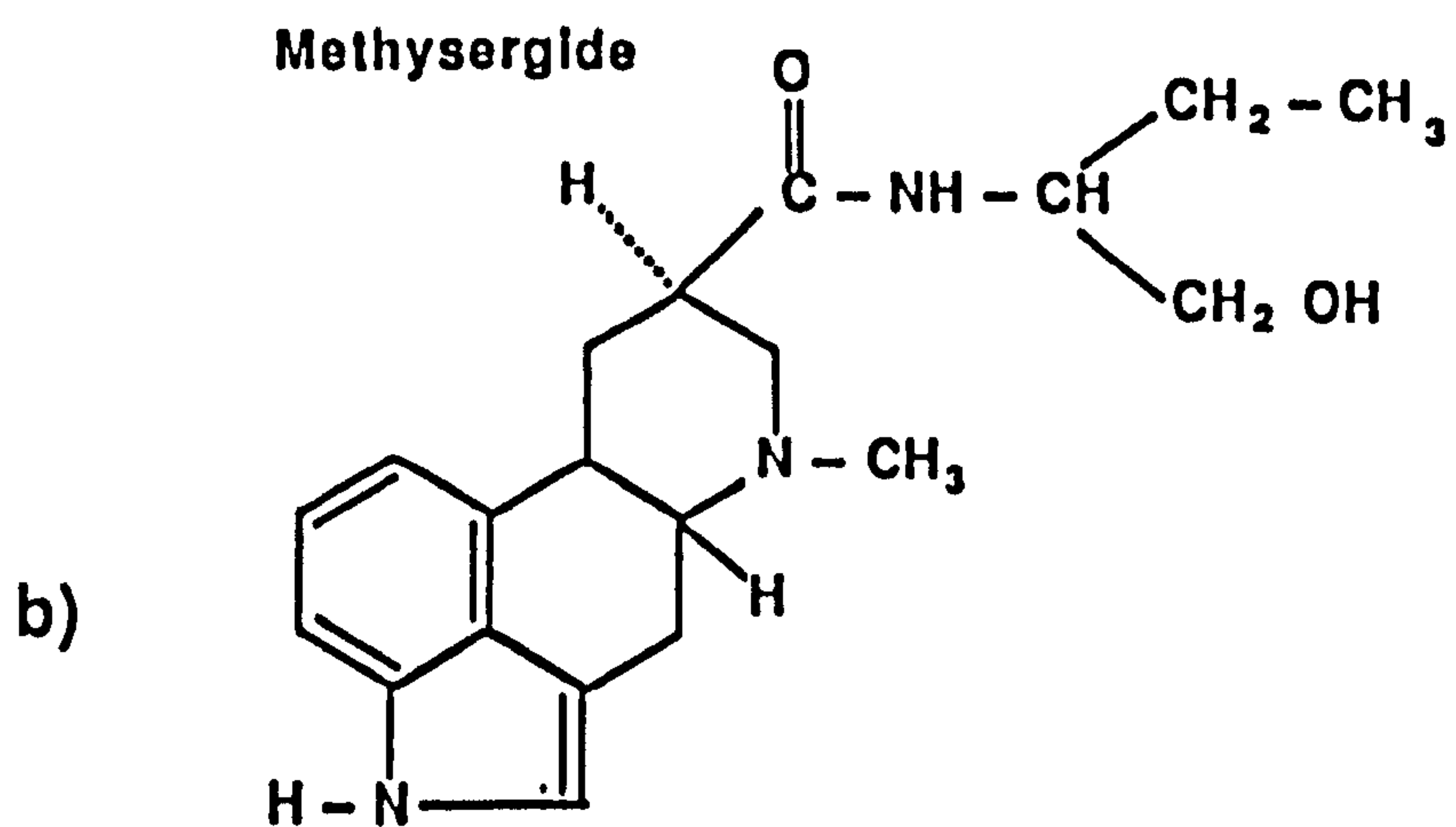
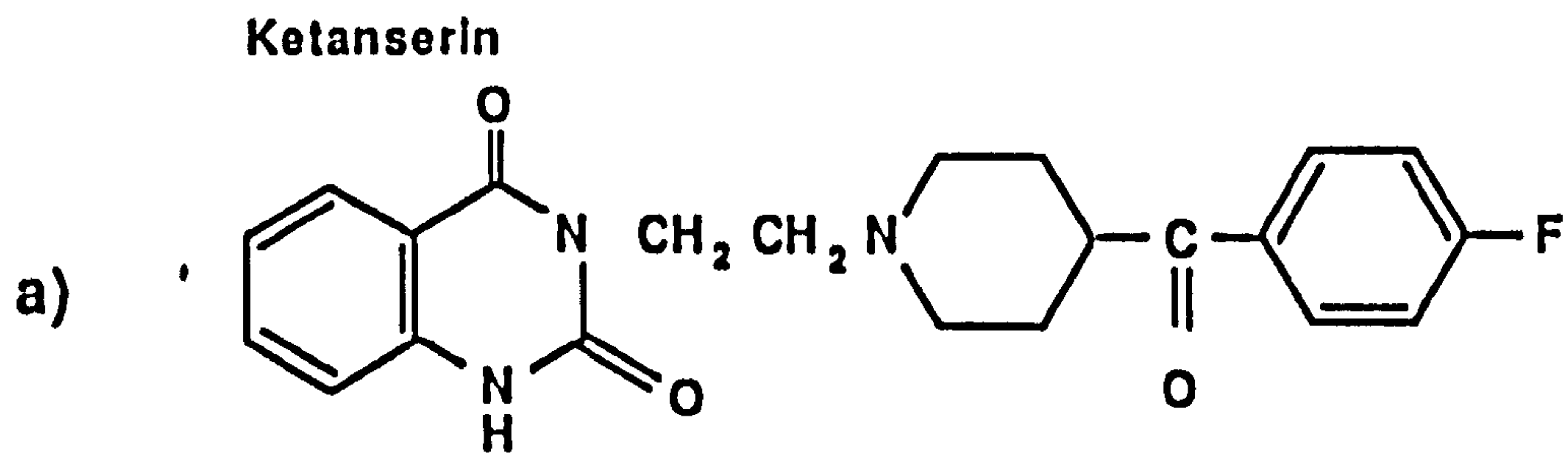
7.2 DRUGS USED IN STUDIES

7.2.1 Ketanserin

Ketanserin was described as a potent type 5HT₂ receptor antagonist in rat cerebral cortex binding studies by Leysen et al 1981. It has a x1000 fold difference in affinity for 5HT₁ and 5HT₂ receptors. The K_i for 5HT₂ = 2.7nM in vasculature, whilst the K_i for 5HT₁ = 2700nM. The reciprocal of K_i is a measure of the binding affinity for the drug, thus the smaller the K_i value, the greater the binding affinity.

In vascular tissue containing 5HT₁ receptors, such as the canine basilar artery, ketanserin and methysergide (below) are non-competitive inhibitors of 5HT. Ketanserin in this case, only has

Fig 7.1 Molecular structure of drugs used



inhibitory effects at concentrations greater than 10^{-6} M. In vessels containing 5HT₂ receptors such as rabbit and rat aorta, both antagonists are competitive, i.e. the antagonism is surmountable at higher concentrations of the agonist (Peroutka et al 1984). At concentrations greater than 10^{-5} M, ketanserin interacts with α_1 and histamine receptors (Leysen et al 1981).

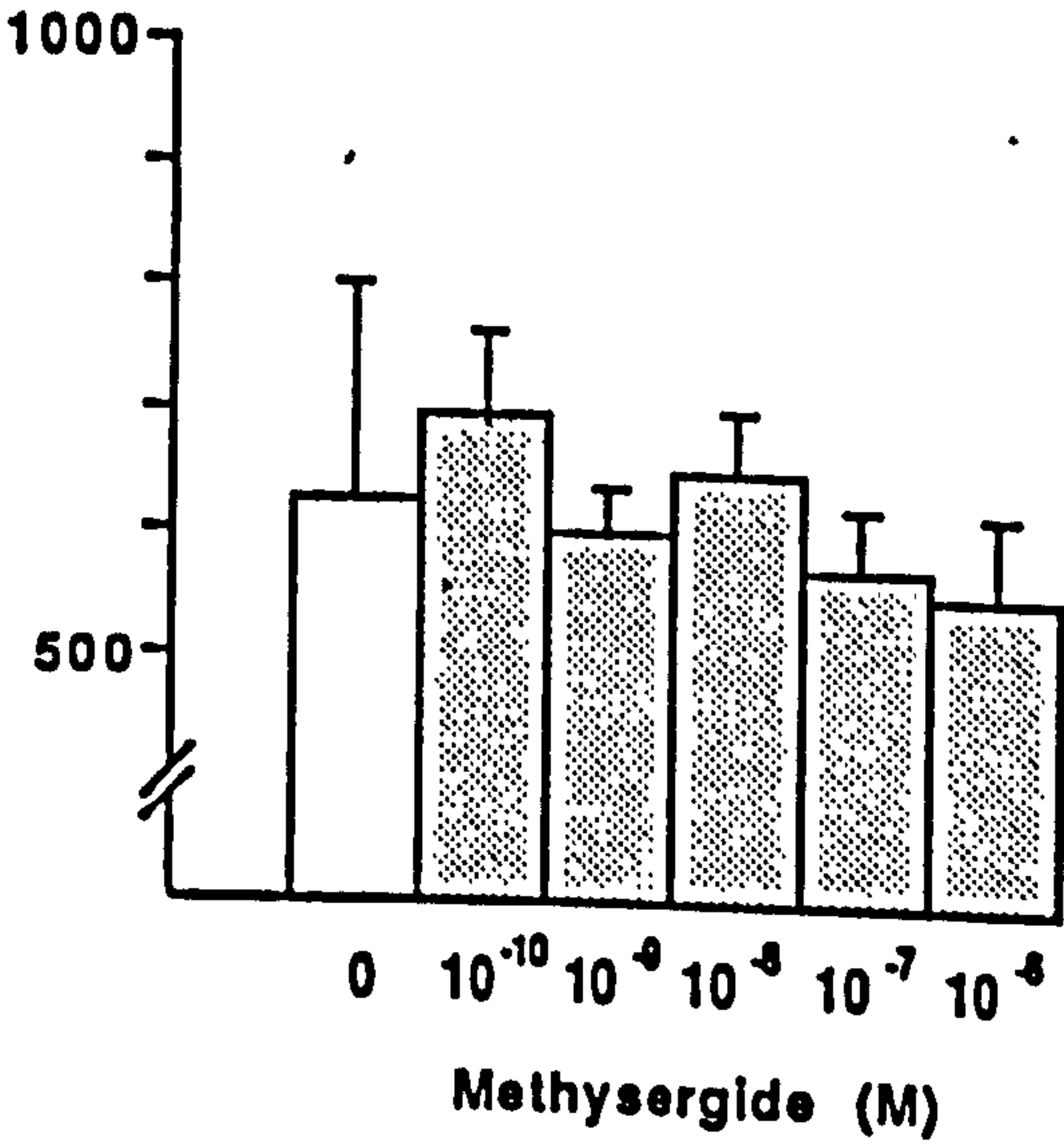
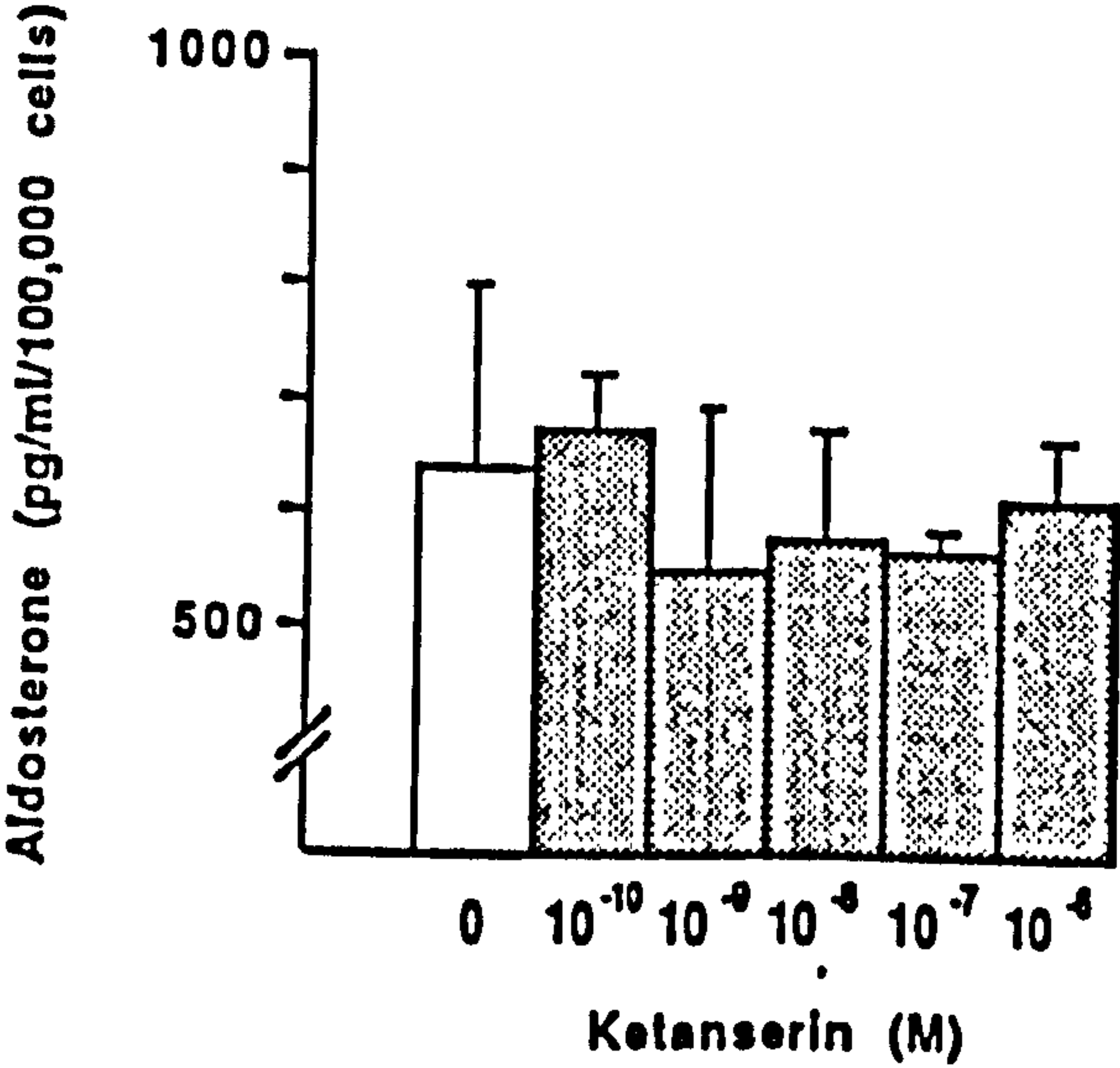
Ketanserin (see Fig.7.1) was obtained from Janssen Pharmaceuticals in ampoules containing 5mg/ml. The molecular weight of the tartrate = 545.5, which has a solubility in water (pH3.4) = 2.4g/100mls.

7.2.2 Methysergide

Methysergide was purchased from Sandoz pharmaceuticals as a white crystalline solid (Deseril) the hydrogen maleate salt $C_{25}H_{31}N_3O_6$ m.wt 469.5.

The natural compound is one of a family of ergot alkaloids. An alkaloid is an organic compound of basic character containing cyclical nitrogen-carbon bonds i.e. pyrole, pyridine or pyrrolidine structures. Ergot is used to describe a subclass of alkaloid compounds derived from a parasitic fungus called ergot, which grows on cereals and especially rye grass. Methysergide is a chemically synthesised compound and like all ergot alkaloids, is an amide of lysergic acid (Fig.7.1(b)).

Fig 7.2 Effects of ketanserin and methysergide on basal aldosterone levels



7.3 STEROID AND cAMP RESPONSE TO 5HT STIMULATION

In the in vitro experiments cAMP, corticosterone and aldosterone output varied for 100,000 cells incubates, and this has been widely reported and interpreted to mean that during cell preparation and aliquotting, cell receptor damage by collagenase digestion, as well as cell dispersion during digestion, may affect the sensitivity and responsiveness of the adrenocortical cells (Vinson et al 1985). Therefore all pooled results were normalised with respect to basal value and results plotted as a percentage of the basal values (Tait et al 1976, 1980a, 1980b); individual experiments were plotted for secretions per 10^5 cells (Aguilera et al 1978a, 1978b).

7.4 RESULTS

7.4.1 The cAMP, Aldosterone and Corticosterone Output with 5HT

Figure 7.2 shows the aldosterone output (pg/ml/ 10^5 cells) and the dose response curves to ketanserin (10^{-10} M- 10^{-6} M) and methysergide.

In both instances, the 5HT antagonists have no significant effect on basal steroid output in the zona glomerulosa cells.

Figure 7.3 shows the effects of ketanserin (10^{-7} M) on the dose response curve to 5HT (10^{-9} M- 10^{-6} M), measuring corticosterone and aldosterone output. Ketanserin at this dose, did not inhibit either the corticosterone or the aldosterone output induced by 5HT, but there was a slight potentiating effect of the drug on both steroids, particularly at higher doses of 5HT.

Fig 7.3 Dose response to 5HT \pm ketanserin 10^{-7} M

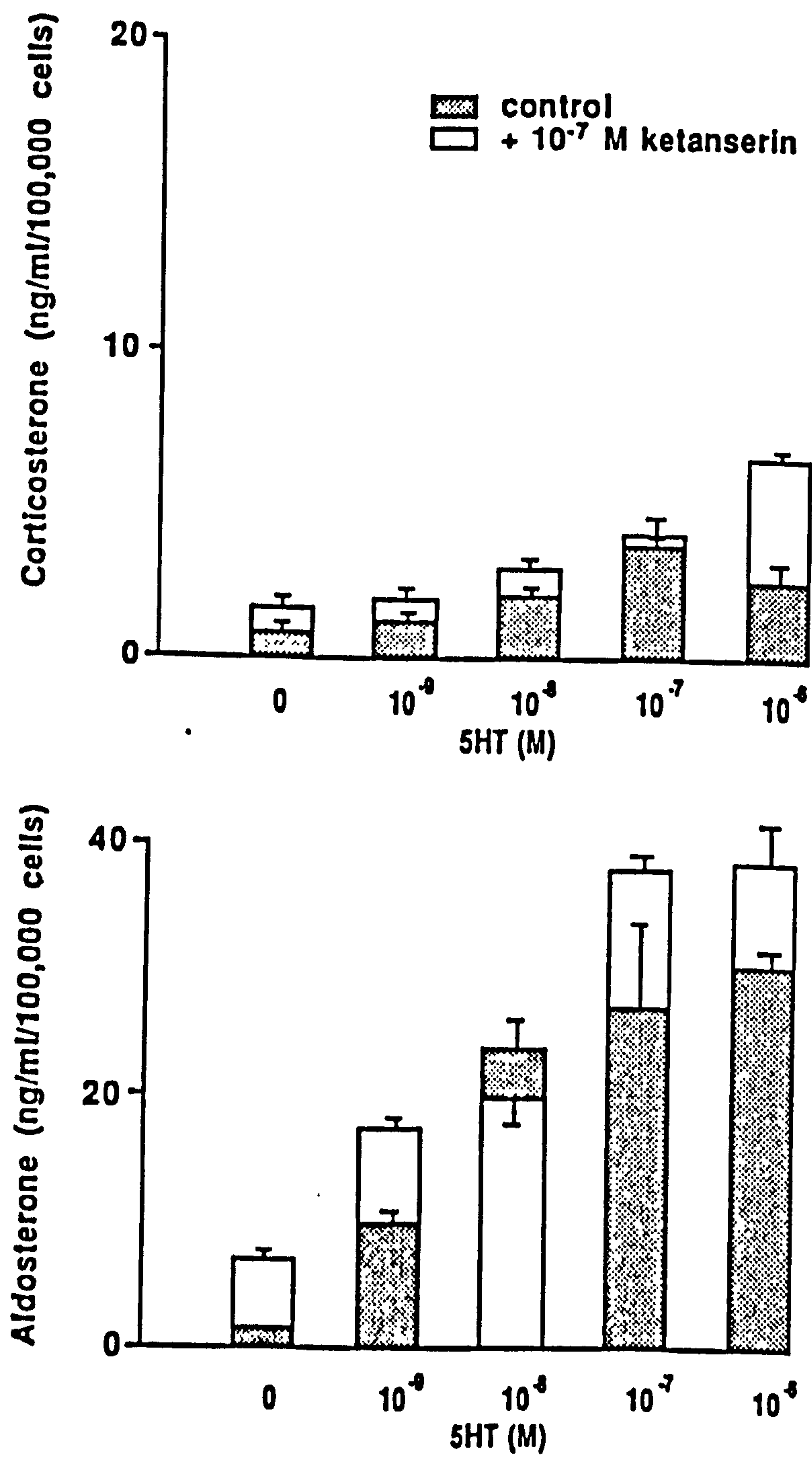


Fig 7.4
% Steroid output for 5HT-stimulated cells $\pm 10^{-7}$ M methysergide

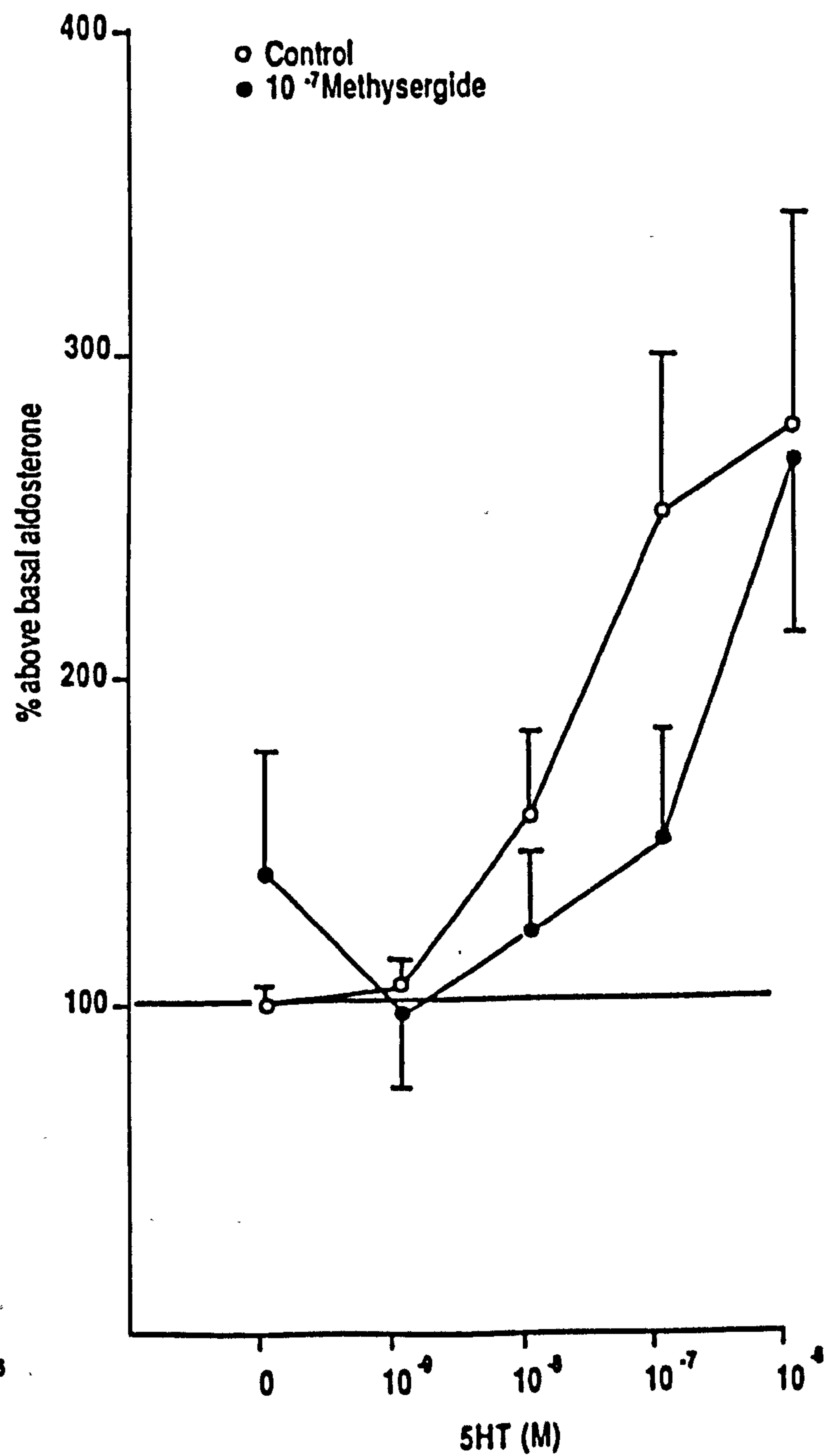
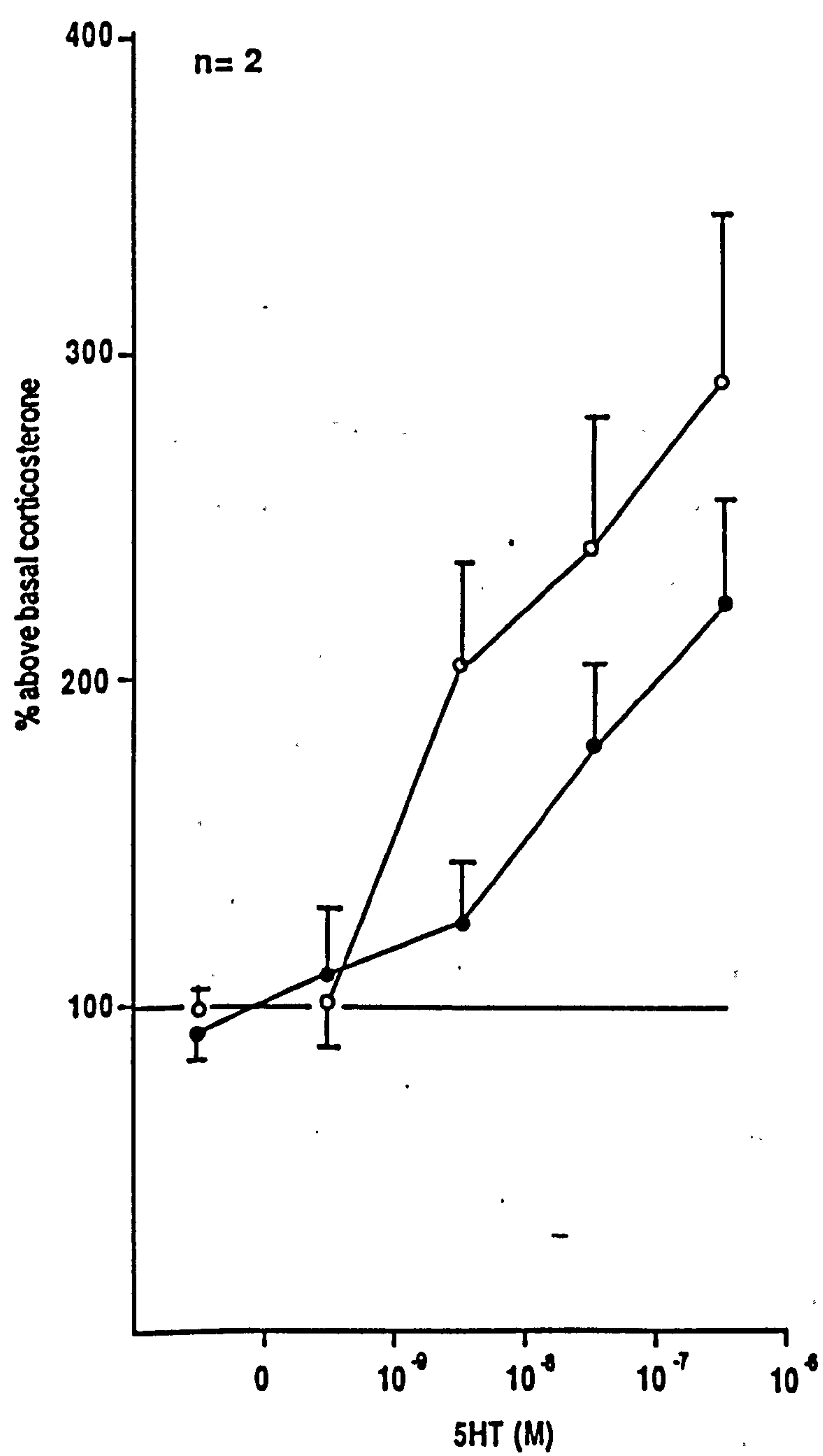


Figure 7.4 shows the percentage above basal values for corticosterone and aldosterone with increasing doses of 5HT in two experiments in triplicate incubations. Methysergide (10^{-7} M) tended to inhibit the dose response curve in a competitive way but this was not statistically significant at this dose.

In Fig.7.5 (A),(B) and (C) are shown the percentage increases in cAMP, corticosterone and aldosterone respectively, for two experiments. Cyclic AMP levels were markedly elevated by 230% above basal, only at 10^{-6} M 5HT. In contrast, corticosterone and aldosterone output was markedly elevated at 10^{-8} M 5HT reaching a maximal of 220% at 10^{-6} M 5HT.

7.4.2 Effects of Ketanserin

However, as further experiments showed, steroid output was not always matched by an increase in cAMP levels. An example of this is shown in Figs 7.6 (A),(B) and (C). Cyclic AMP levels were not elevated at any doses of 5HT whereas corticosterone and aldosterone were significantly increased in this experiment at 10^{-9} M 5HT. In the same experiment, ketanserin (10^{-6} M) inhibited aldosterone and corticosterone output at 10^{-8} M and 10^{-7} M 5HT. Discrepancies occurred in ketanserin's inhibitory effects on corticosterone at 10^{-6} M 5HT without affecting aldosterone at the same concentration; conversely, ketanserin blocked aldosterone output at 10^{-9} M 5HT and not corticosterone output. This was a common finding in all experiments measuring the inhibitory effects on both corticosterone and aldosterone but because aldosterone is produced solely by the

Fig 7.5 cAMP, corticosterone and aldosterone output with 5HT stimulation

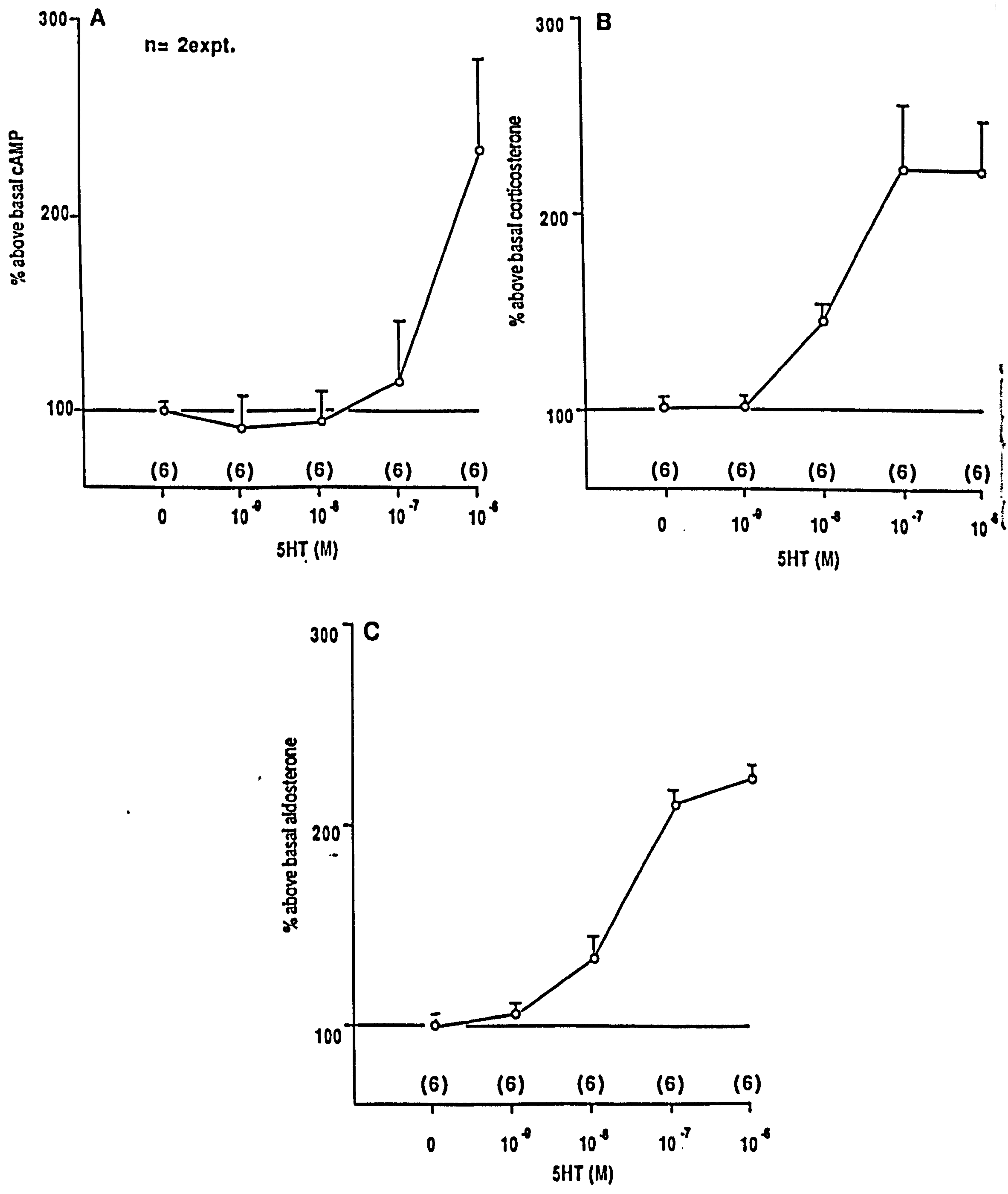


Fig 7.6 Effect of 10^{-6} M ketanserin on cAMP, corticosterone and aldosterone output with 5HT stimulation

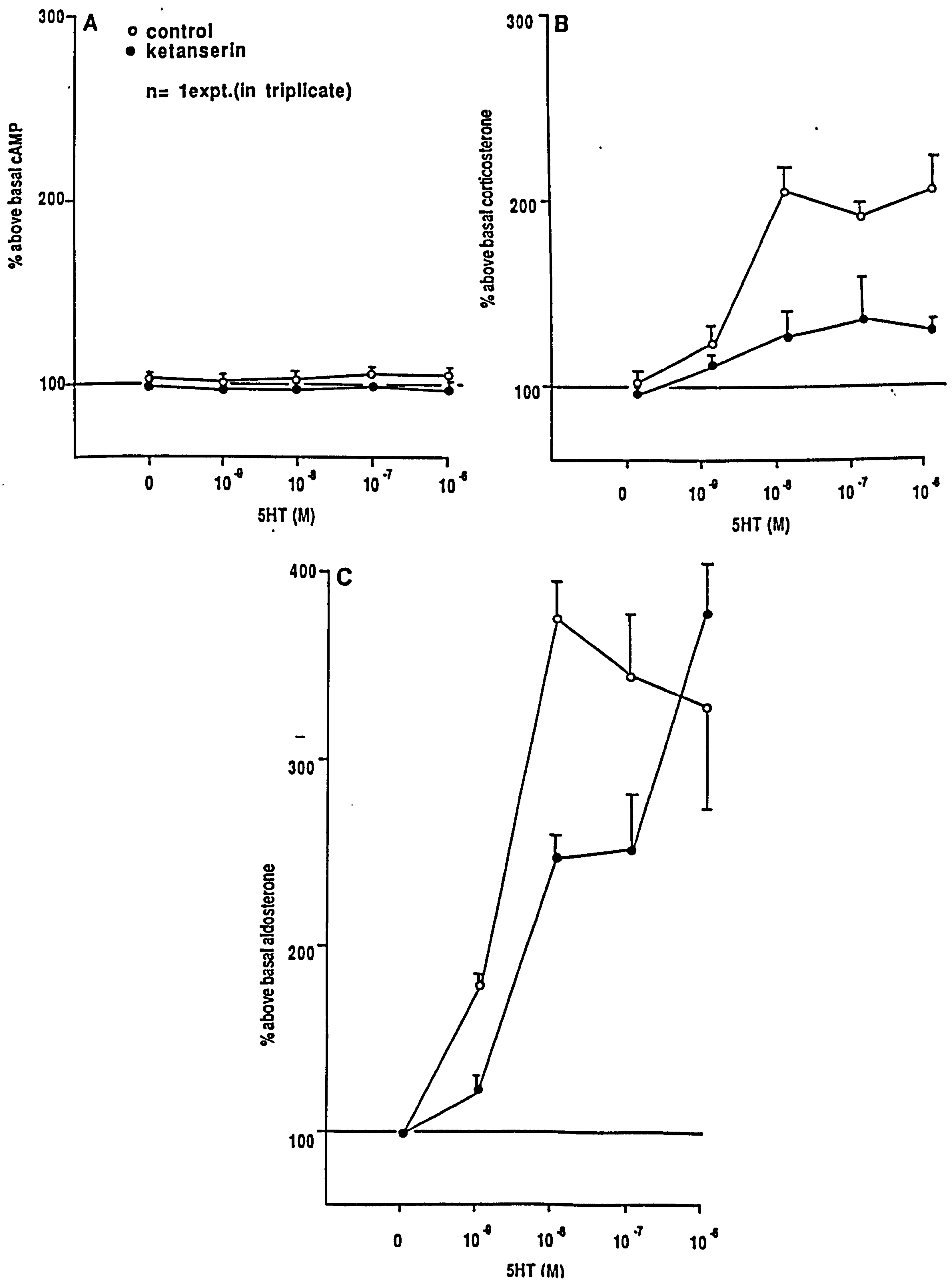
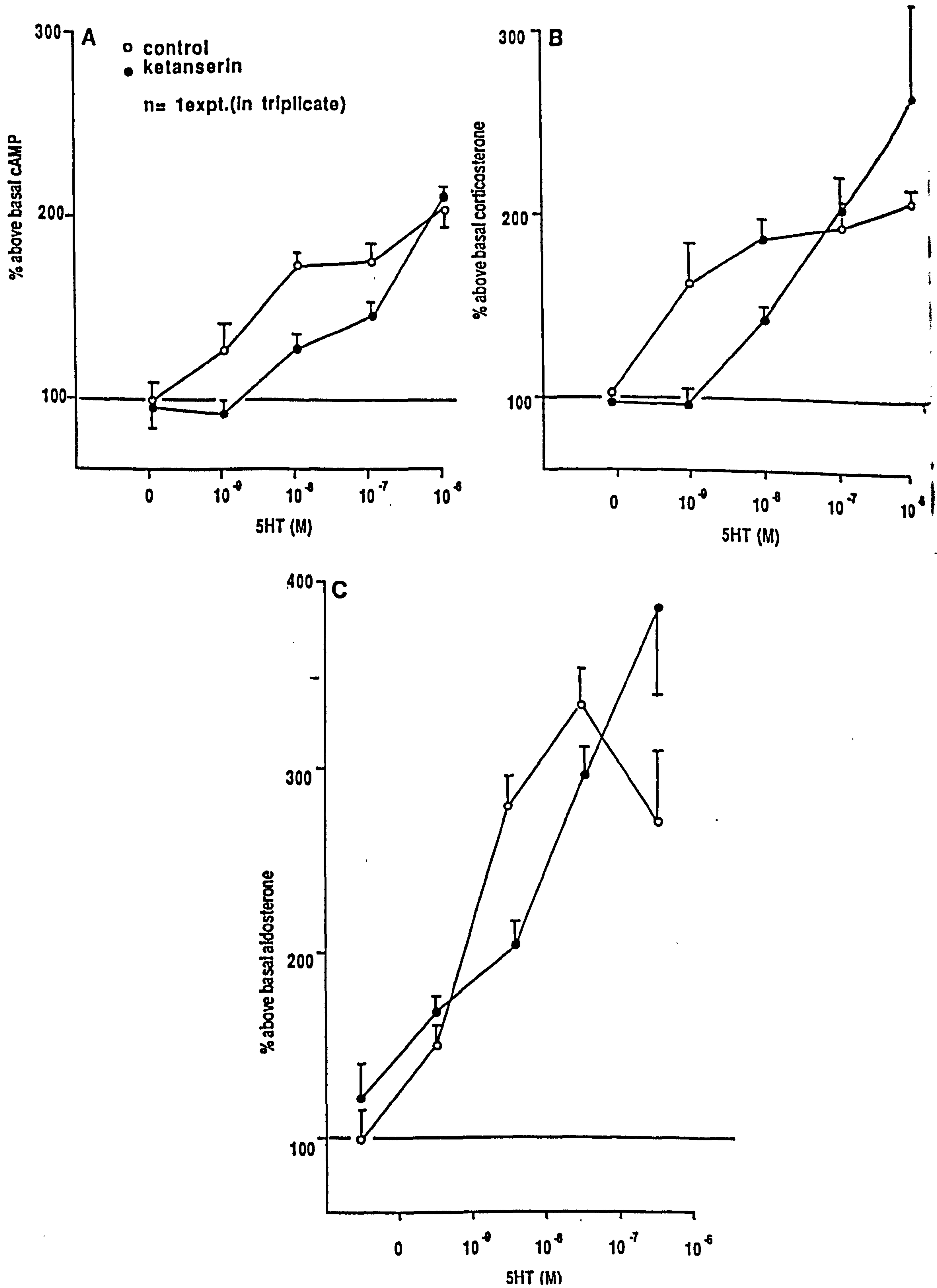


Fig 7.7
Effect of 10^{-6} M Ketanserin on cAMP, corticosterone and aldosterone output with 5HT stimulation



zona glomerulosa, this was considered the more important parameter to use in measuring the potency of the 5HT antagonists.

In Fig.7.7 (A) cAMP levels were elevated at all doses of 5HT greater than 10^{-9} M and the percentage values closely paralleled corticosterone output at 10^{-8} M- 10^{-6} M 5HT. Ketanserin significantly inhibited cAMP production at 10^{-8} M- 10^{-7} M 5HT. The aldosterone percentage increase in the control incubates were markedly greater than the cAMP response curve and inhibition by ketanserin was significant but not as great as cAMP inhibition.

As stated in Chapter 5, ketanserin at doses $\geq 10^{-5}$ M in vascular and brain tissue, has affinity for α_1 receptor sites. In the experiments shown in Figs. 7.8 and 7.9 the effects of phentolamine (a non-specific α antagonist) and prazosin (an α_1 antagonist) at 10^{-6} M, were assessed for their inhibitory effects on 5HT stimulated steroidogenesis in the zona glomerulosa.

7.4.3 EFFECTS OF ALPHA ANTAGONISTS

Phentolamine

The drug was purchased from Ciba Laboratories in ampoules containing 10mg/ml. The molecular weight = 377.5 and the structure is shown in Fig.7.1(c). Stock solutions were prepared by adding 25 μ l of the drug to 6.6mls of incubation buffer = 1mM stock.

Prazosin

The drug was purchased from Pfizer Ltd., as the white crystalline

hydrochloride salt (HYPOVASE) m.wt $C_{19}H_{22}ClN_5O_4$ and dissolved in DMSO at a final concentration of 0.01% in the incubation medium. As in the antiserotonergic drug experiments, cells were incubated with the drugs alone for 15 mins before addition of the agonist. Figures 7.8 (A), (B) and (C) show the dose response to 5HT + prazosin, for cAMP, corticosterone and aldosterone in two pooled experiments with data normalised. As previously seen, cAMP levels were only elevated at $10^{-6}M$ 5HT, whereas corticosterone and aldosterone output were elevated at $10^{-8}M$ 5HT. Preincubation with prazosin ($10^{-6}M$) did not significantly affect basal and stimulated corticosterone and aldosterone, but cAMP levels though depressed with prazosin present, were not significantly depressed (Students paired t-test $p < 0.05$).

Figure 7.9 shows the results from one experiment done in triplicate in the presence of $10^{-6}M$ phentolamine. In Fig.7.9(A) cAMP levels were significantly elevated at $10^{-7}M - 10^{-6}M$ 5HT. The results with phentolamine suggested no inhibition of cAMP output compared to unstimulated outputs with increasing doses of 5HT in the control curve. In Fig.7.9 (B) and (C), both corticosterone and aldosterone were significantly elevated at all doses of 5HT; phentolamine did not inhibit these responses. However, the cAMP curve did not parallel the steroid response curves in the control experiment.

7.4.4 Effects of Methysergide

Methysergide was preincubated with the cells at $10^{-6}M$ for 15 mins before addition of 5HT to the cells.

In Fig. 7.10 (A) cAMP levels were significantly elevated at $10^{-7}M$

Fig 7.8 Effect of 10^{-6} M prazosin on cAMP, corticosterone and aldosterone output with 5HT stimulation

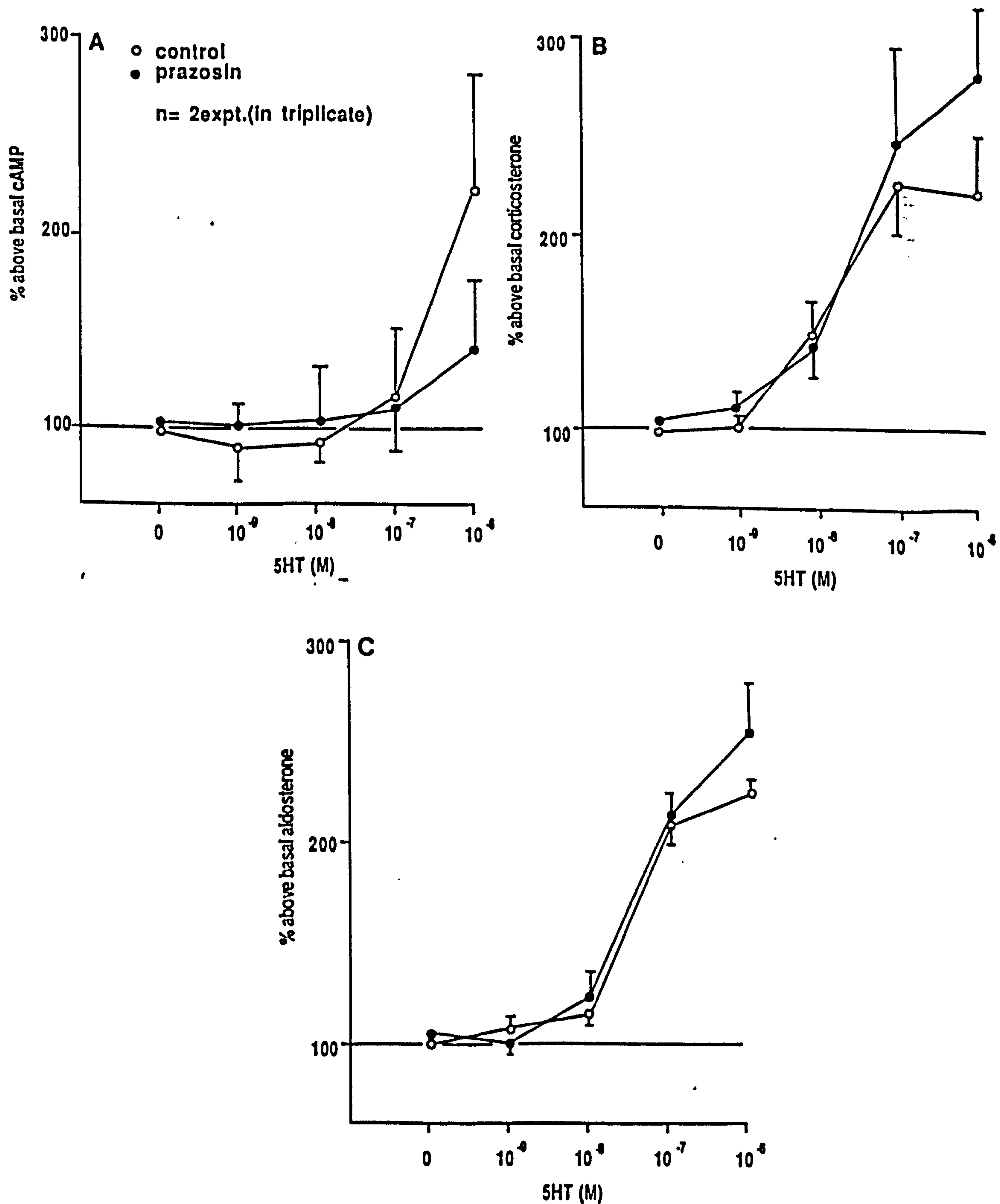


Fig 7.9 Effect of 10^{-6} M phentolamine on cAMP, corticosterone and aldosterone output with 5HT stimulation

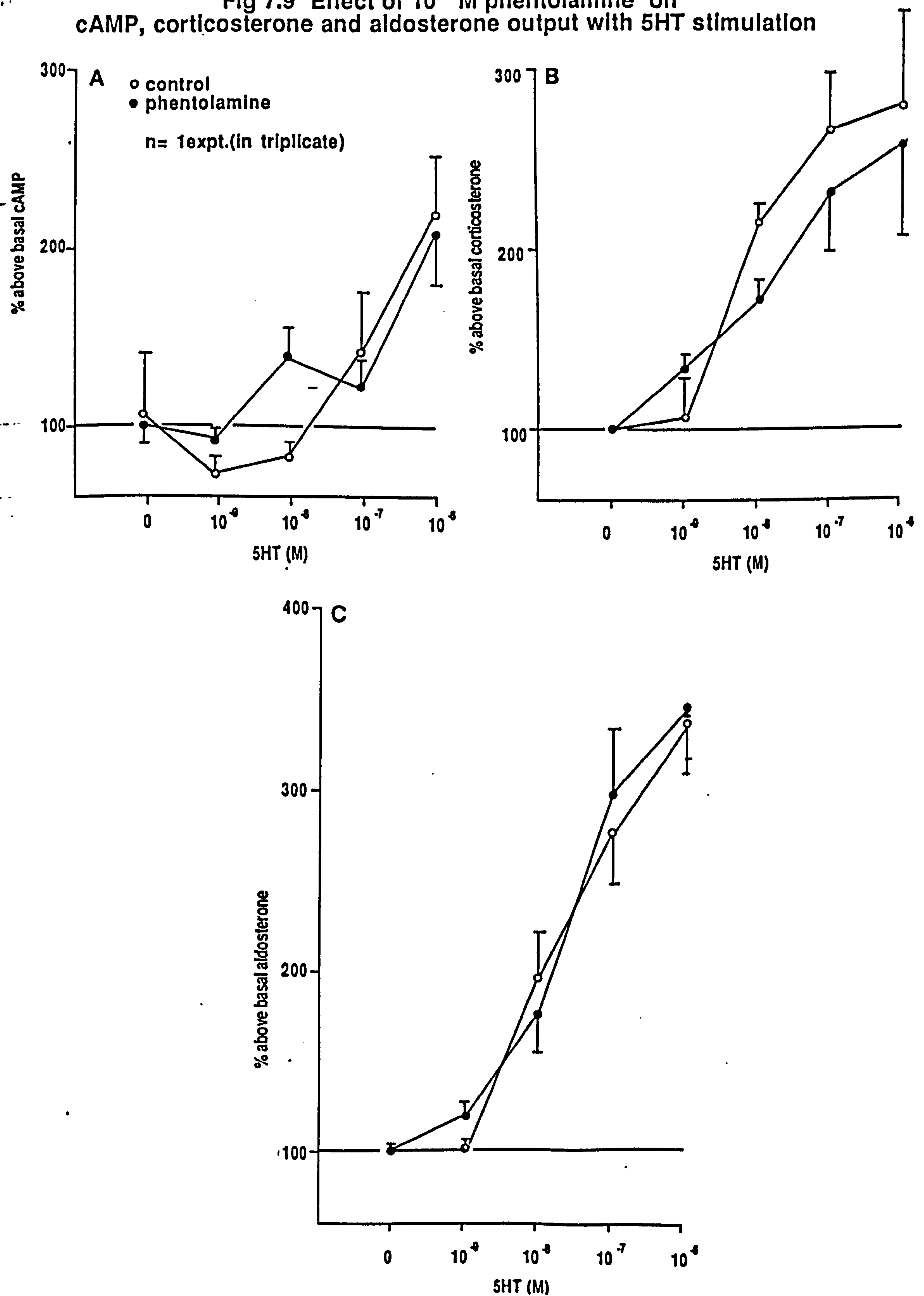
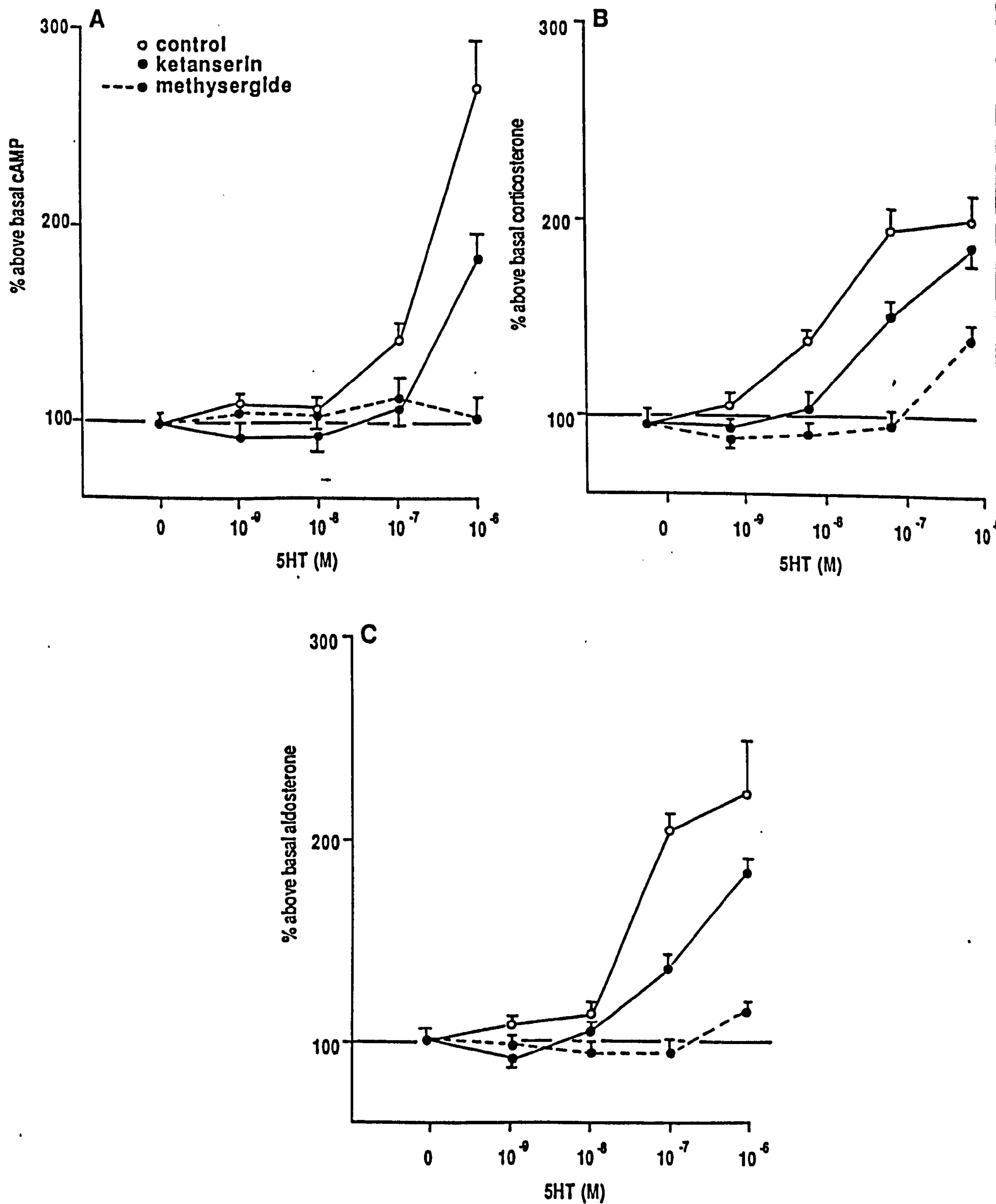


Fig 7.10 Effect of 10^{-6} M methysergide and 10^{-6} M ketanserin on cAMP, corticosterone and aldosterone output with 5HT stimulation



and 10^{-6} M 5HT and in (B) and (C) corticosterone and aldosterone respectively, were increased at 10^{-8} M 5HT. Methysergide was more potent than ketanserin in inhibiting the cAMP, corticosterone and aldosterone output at all doses of 5HT. Methysergide inhibited cAMP production at all doses of 5HT that stimulated its production. Figure 7.11(A) shows that 5HT stimulates cAMP at lower doses, suggestive of closer coupling in this experiment, although the percentage increase in cAMP was not as great as the increase in steroid output. The same observation was made in other experiments. The ED_{50} i.e. the dose of 5HT that stimulates 50% of the maximal response, for cAMP was on average 10^{-7} M compared to an ED_{50} of 10^{-8} M for corticosterone and aldosterone secretion. In Fig. 7.12(A) methysergide (10^{-6} M) did not inhibit cAMP at 10^{-6} M 5HT although B output was depressed in a non-competitive way and aldosterone was significantly decreased, since increasing doses of 5HT did not overcome the inhibition.

7.5 DISCUSSION

7.5.1 Effects of Ketanserin

From the ketanserin data, the lack of inhibition with the alpha antagonists (phentolamine and prazosin) tends to suggest that 5HT is not interacting with α receptors but rather the effects of ketanserin are due to its antiserotonergic properties.

In the ketanserin experiments, cAMP was not always increased although steroid was significantly elevated. However, ketanserin

Fig 7.11

Effect of 10^{-6} M Methysergide on
cAMP, corticosterone and aldosterone output with 5HT stimulation

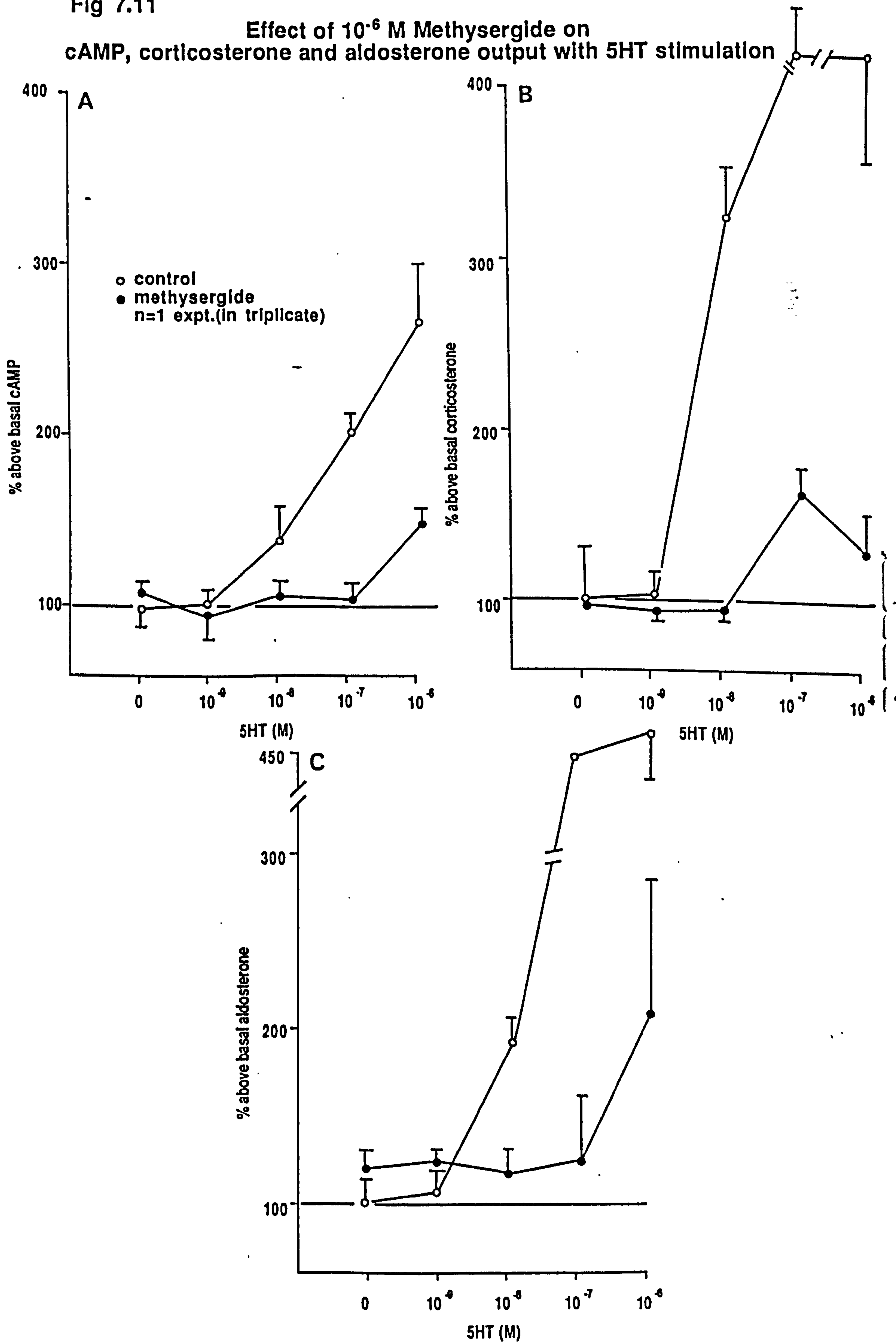
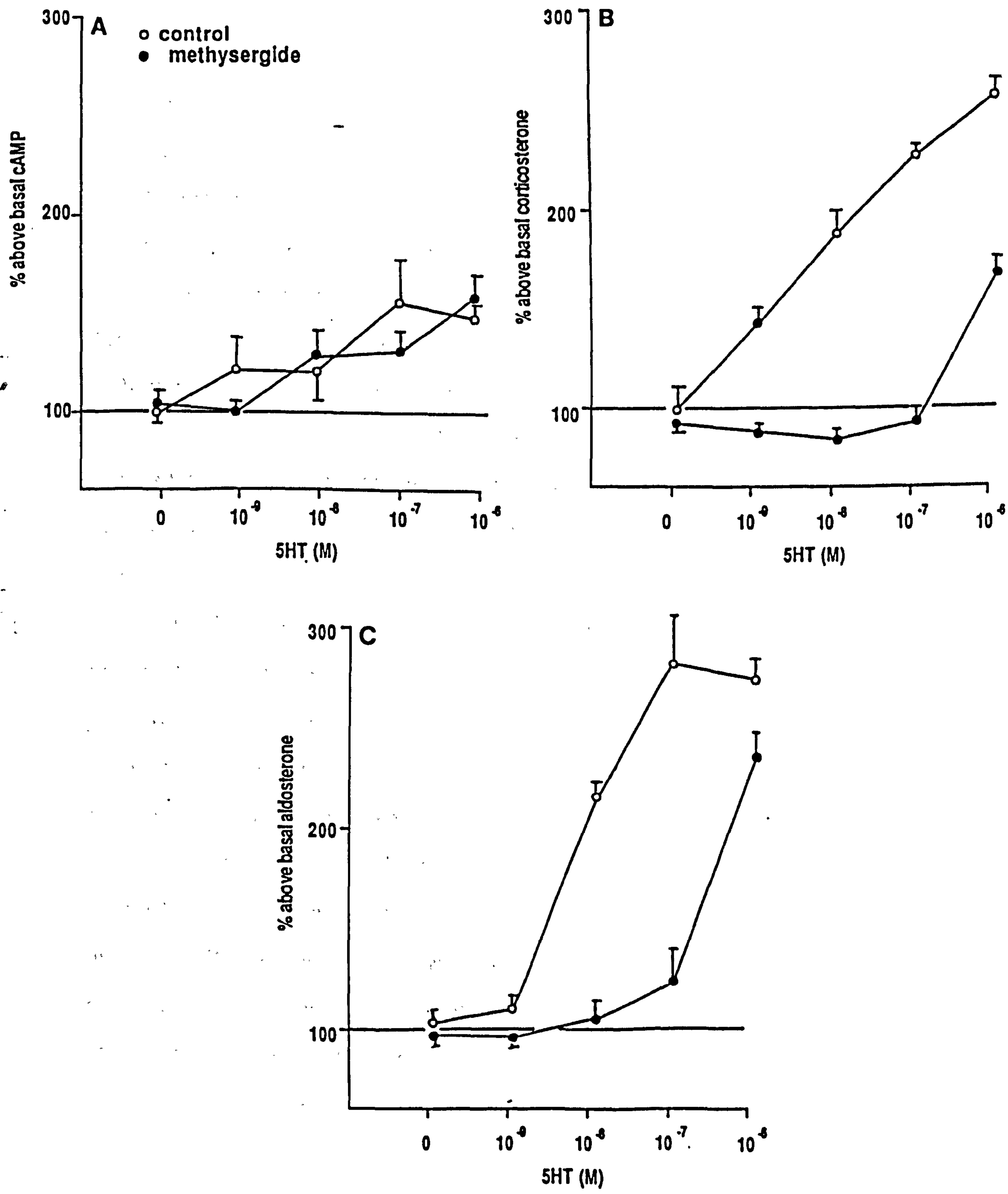


Fig 7.12 Effect of 10^{-6} M methysergide on cAMP, corticosterone and aldosterone output with 5HT stimulation



blocked steroid production and when cAMP was increased, also inhibited its production. There appears to be a clear dissociation between 5HT's effect on stimulating steroid secretion and cAMP production. Its stimulation of cAMP appears to be a loosely coupled event, more apparent at higher doses of 5HT i.e. 10^{-6} M (e.g. Fig. 7.5) but is not a prerequisite in the production of steroid (e.g. Fig. 7.6). As previously stated, ketanserin interacts with type 5HT₂ receptors, which are not thought to be linked to adenylate cyclase. However Conn (1986) suggested that 5HT₂ receptor binding of 5HT may require GTP, which suggested some adenylate cyclase interaction. If 5HT is interacting with an adrenal 5HT receptor, it appears to be loosely coupled to adenylate cyclase, which may be 'channeled' into steroid production (Schulster et al., 1980) or equally the possibility exists that 5HT spare receptors may exist which at increasing doses of 5HT (notably 10^{-6} M), may be activated, and together may augment adenylate cyclase activation as described for ACTH receptors (Buckley et al., 1981). The possibility exists that 5HT stimulates steroid through Ca^{2+} mediation as well as/or in preference to cAMP production, and is blocked by ketanserin. In this regard, 5HT stimulated steroidogenesis appears to fit into the receptor model of control of cellular function termed 'monodirectional control' (Takai et al., 1985) in that the stimulus, in this case 5HT, elicits production of several second messengers e.g. Ca^{2+} and cAMP that are stimulated independently and act as positive modulators of the stimulated response (see Rasmussen and Barrett 1984) although in this

case, cAMP may not be of great importance since cAMP did not always follow steroid inhibition. The other mode of control is termed 'bidirectional control' in that the second messenger modulates the activity in a negative way, of other potential messengers. As discussed in the chapter on 5HT receptors, 5HT₂ receptors are likely candidates for a prostacyclin mediated increase in steroid secretion. However, in brain and vascular tissues, ketanserin is a more potent competitive inhibitor at lower concentrations (nM range) than was seen in these studies. Additionally, ketanserin did not appear to inhibit steroid production by 5HT in a competitive way, so the likelihood that type 5HT₂ receptors exist in the adrenal tissue is remote (see later for specificity effects).

7.5.2 Effects of Methysergide

In the experiments with methysergide at 10^{-6} M, cAMP was not always inhibited, although the antagonist always significantly inhibited steroid production. Here again the antagonist inhibits 5HT mediated steroidogenesis but cAMP does not appear to be an essential second messenger in steroid secretion. As indicated earlier, methysergide at the lower dose of 10^{-7} M in two pooled experiments, did not significantly (Students paired t-test $p < 0.05$) inhibit corticosterone or aldosterone secretion (Fig.7.4). Again, the dose of antagonist used is in the range described for interaction with type 5HT₁ receptors. At this point, it may be appropriate to mention that agonists including the indoleamines, are more potent at interacting with type 5HT₁ receptors, which also have lower affinity for 5HT antagonists. In Chapter 5 it was shown that 5HT had a potent

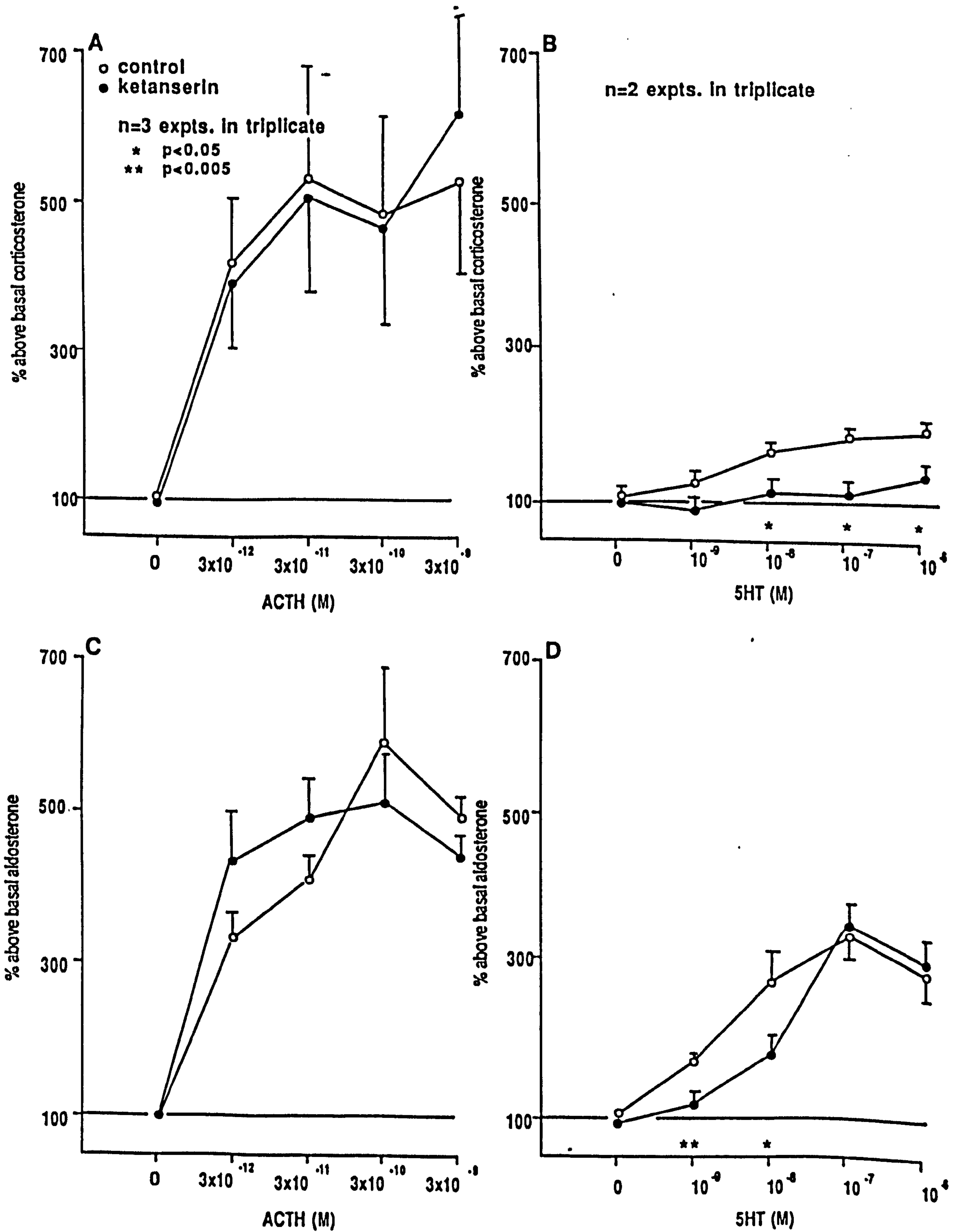
effect - ED₅₀ approximately 5×10^{-9} M - on aldosterone biosynthesis (Tait 1975). The evidence tends to favour a type 5HT₁ subtype, which may coincidentally associate with adenylate cyclase but even without cAMP involvement, still elicit a 5HT stimulated steroid response. Nelson (1980), found that in brain tissue, 5HT antagonists were potent at displacing [³H]-5HT from 5HT binding sites and had little or no effect in inhibiting 5HT stimulated cAMP production. They also found that 5HT had a K_d 1-2nM in terms of binding, whereas 5HT stimulated cAMP production had a K (apparent) 0.5μM-1μM. It was suggested that 5HT could bind to a heterogenous set of receptors some of which are coupled to cAMP production. The stimulatory effects of indolamines agonists were also investigated, including bufotenine and 5-methoxy-N,N-dimethyltryptamine, which displaces [³H]-5HT from brain membranes and activates adenylate cyclase in contrast to piperazine type compounds which only affect [³H]-5HT binding. This suggests structural differences in the adenylate cyclase receptor compared to the [³H]-5HT binding site. Shah (1979) demonstrated that 5HT over a concentration range of 10^{-7} M - 10^{-3} M could specifically stimulate cAMP production in rat renal cortex. Serotonin has also been shown to stimulate cAMP in vascular endothelial tissues and insect salivary glands (Shama et al., 1979., Berridge et al., 1979). However, in other tissues, e.g. bronchial smooth muscle, platelets, uterine and mammalian umbilical artery, 5HT increases cGMP without affecting cAMP levels. In these studies, only cAMP was looked at so the possible involvement of other messengers e.g. Ca²⁺, PI and cGMP, could not be discerned. Fozard (1983a) discussed the anomalies in

5HT receptor identification and possible association with cAMP formation. It was shown that type 5HT₁ receptors have sometimes been linked with adenylate cyclase but have met with doubts as raised above. There have been claims that 5HT₁ recognition sites have similar properties to the 5HT inhibitory autoreceptor located on presynaptic 5HT neurone sites, since metitepine is an effective antagonist at the autoreceptor and displaces [³H]-5HT with a similar potency. Methysergide however has no effect at the autoreceptor even at μ M concentrations, yet displaces [³H]-5HT from its binding site with an IC₅₀ of 62nM. Similarly, metergoline, another 5HT antagonist, has no effect on the autoreceptor. As revealed in recent studies in binding properties of several 5HT agonists and antagonists, the 5HT₁ receptor represents a multiple class of binding sites, all of which bind [³H]-5HT and interact with indole agonists, but may not all mediate their physiological effects through the generation of cAMP. The same could apply in these initial studies in the adrenal cortex, although extensive pharmacological studies with many agonists and antagonists would be needed in further investigations.

7.6 SPECIFICITY OF 5HT ANTAGONISTS

Further antagonism studies to characterise a 5HT receptor in the adrenal, as described in other tissues using a range of doses to the antagonist or several antagonists, was not attempted at this stage since the specificity of the antagonism was sought. Dose response curves to ACTH, AII and K⁺ in the presence of the antagonists

Fig 7.13 Effect of 10^{-6} M ketanserin on corticosterone and aldosterone output with 5HT and ACTH stimulated glomerulosa cells



methysergide and ketanserin (10^{-6} M), were carried out using M199 (0.2%BSA and 0.2% glucose). Only corticosterone and aldosterone were measured by RIA as described previously.

7.6.1 RESULTS

7.6.2 Effects of Ketanserin on the Dose Response Curve to ACTH

Figure 7.13 shows that ketanserin had no effect on corticosterone and aldosterone output in ACTH stimulated steroidogenesis in the same experiments in which ketanserin significantly inhibited aldosterone output at 10^{-9} M and 10^{-8} M 5HT-stimulated steroidogenesis.

7.6.3 Ketanserin in K^{+} -stimulated Steroidogenesis

Figure 7.14 shows that all doses of K^{+} , ketanserin did not inhibit steroid output, although 5HT-stimulated corticosterone output was significantly inhibited at 10^{-9} M - 10^{-7} M and aldosterone output was inhibited at all doses of 5HT ($p < 0.05$).

7.6.4 Ketanserin in AII-stimulated Steroidogenesis

Figure 7.15 shows that ketanserin significantly inhibited the AII stimulated aldosterone response at 10^{-8} M ($p > 0.05$) and at all doses greater than 10^{-11} M AII, ketanserin significantly inhibited corticosterone output. In the same experiments, ketanserin inhibited 5HT stimulated aldosterone output only at 10^{-8} M - 10^{-7} M 5HT. In a number of other experiments, ketanserin inhibited the AII response curve at higher AII concentrations and only affected the lower doses of 5HT-stimulated steroidogenesis (e.g. Figs 7.14 and 7.15).

Fig 7.14 Effect of 10^{-6} M ketanserin on corticosterone and aldosterone output with 5HT and K^{+} -stimulated glomerulosa cells

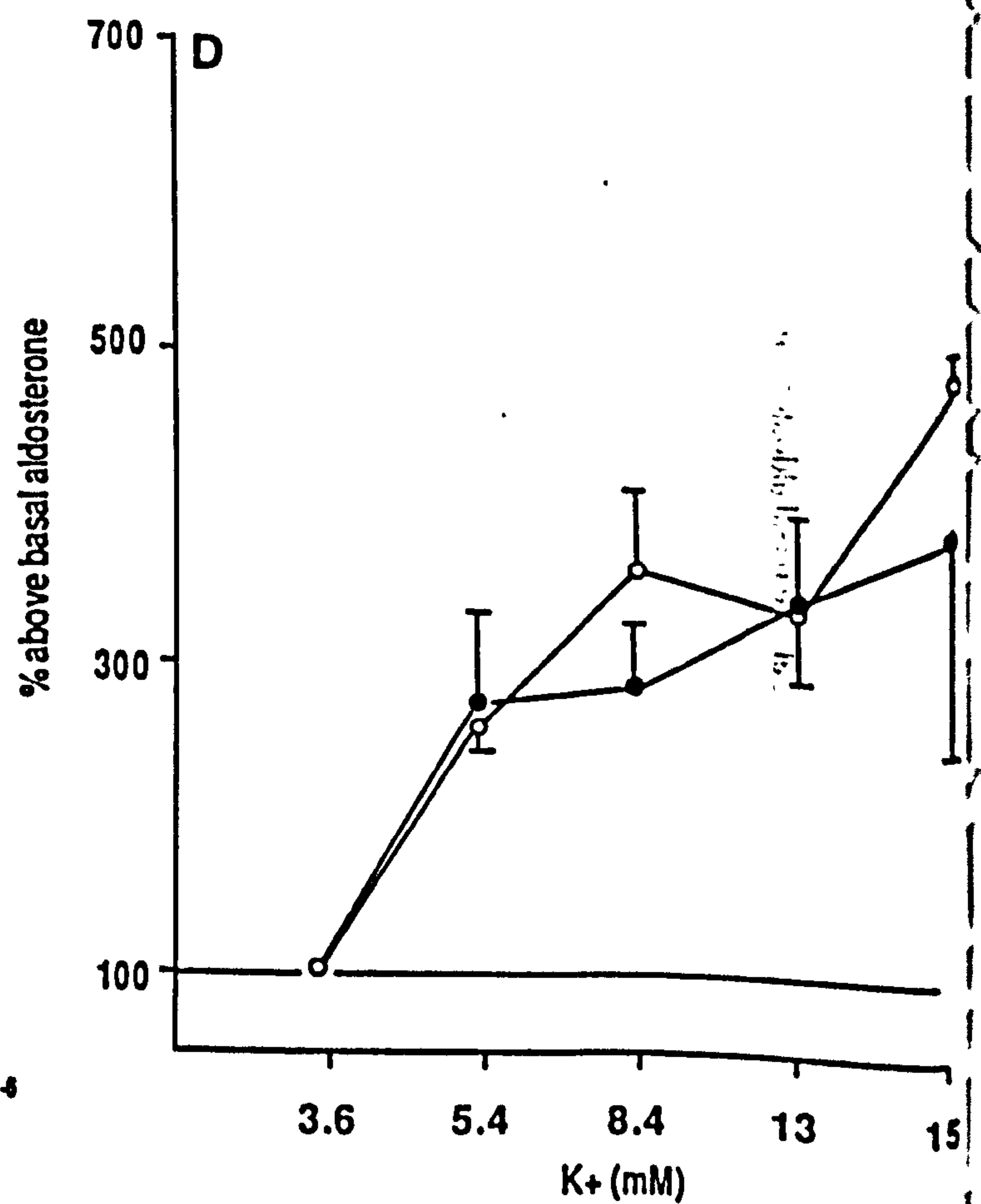
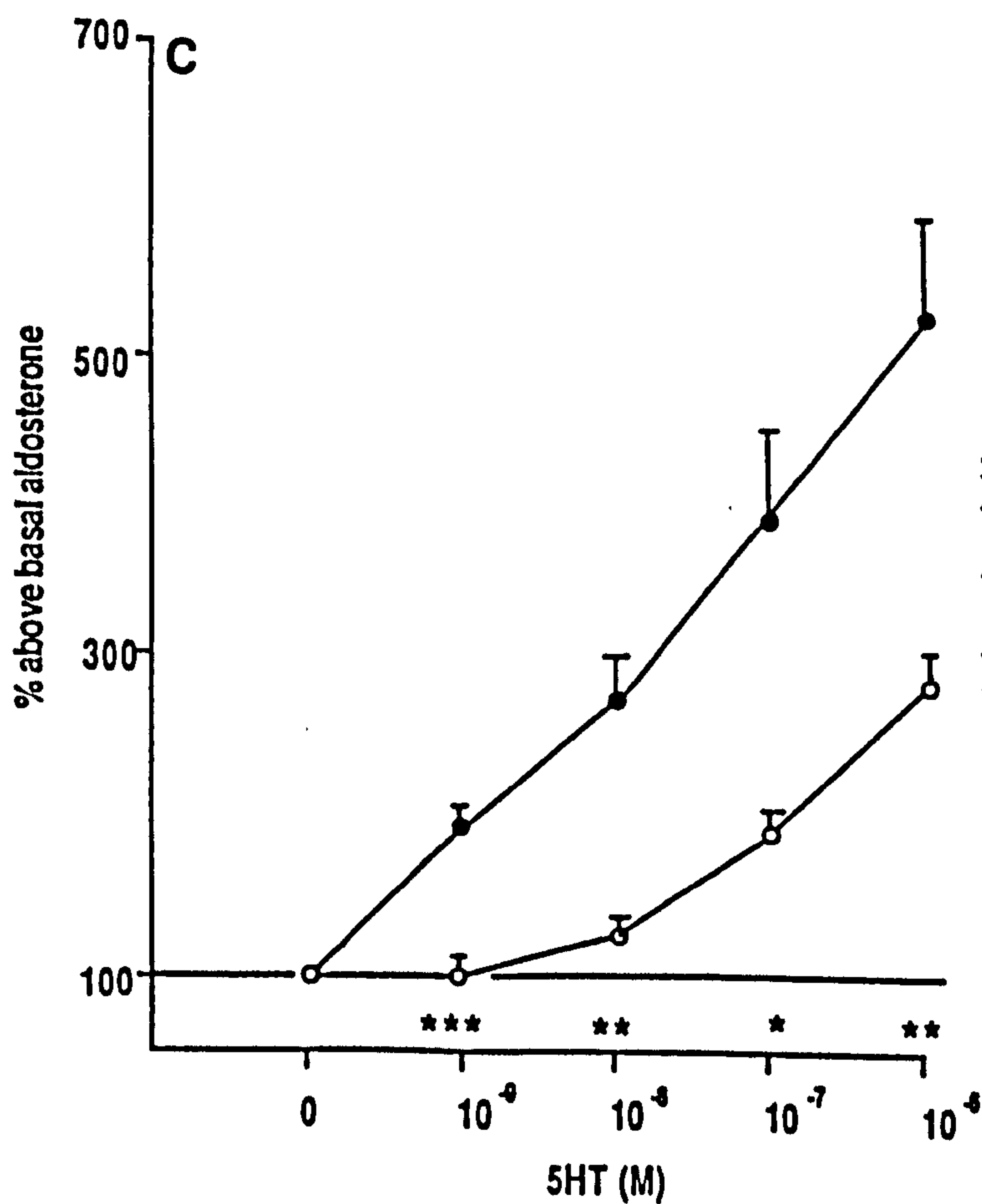
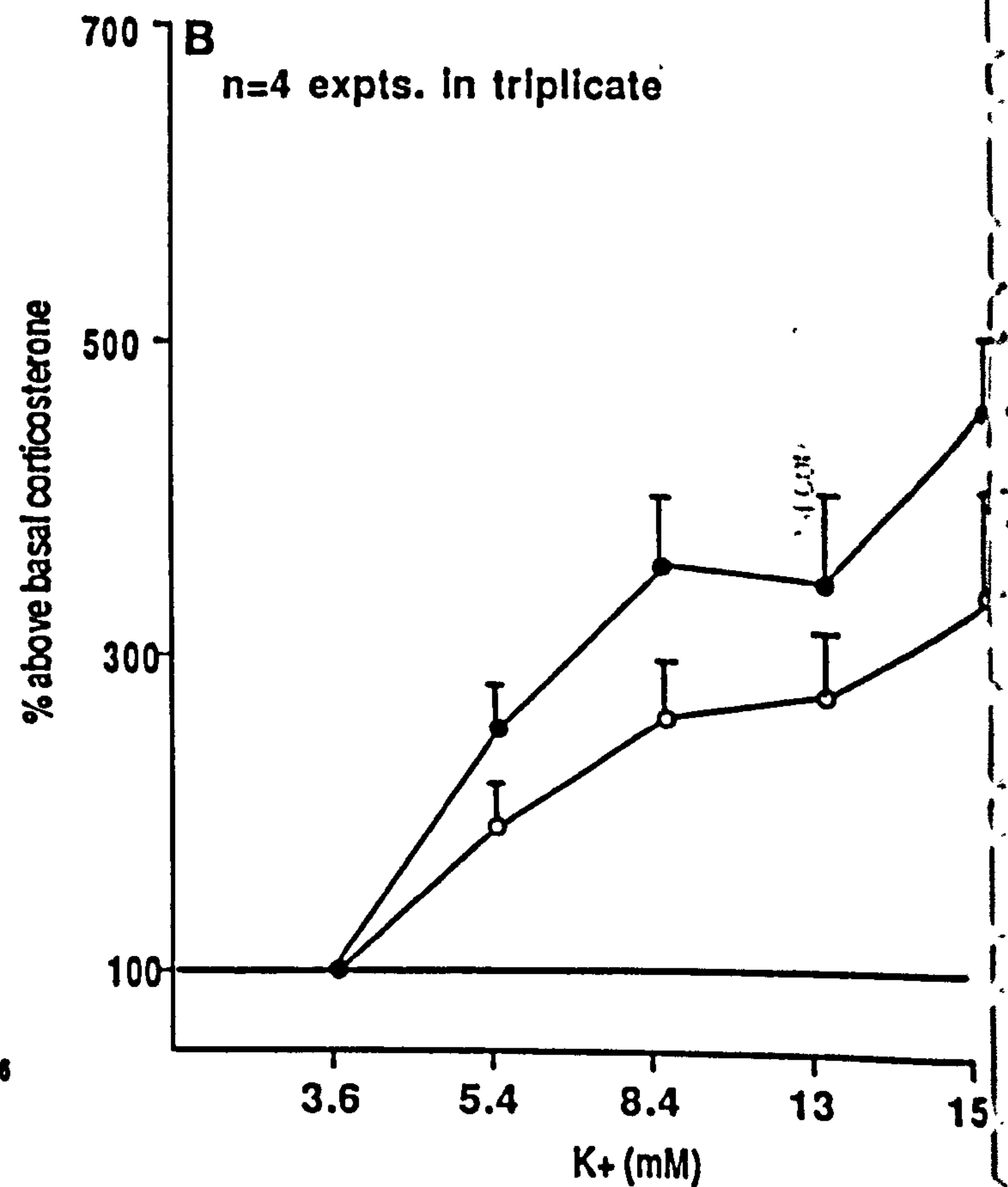
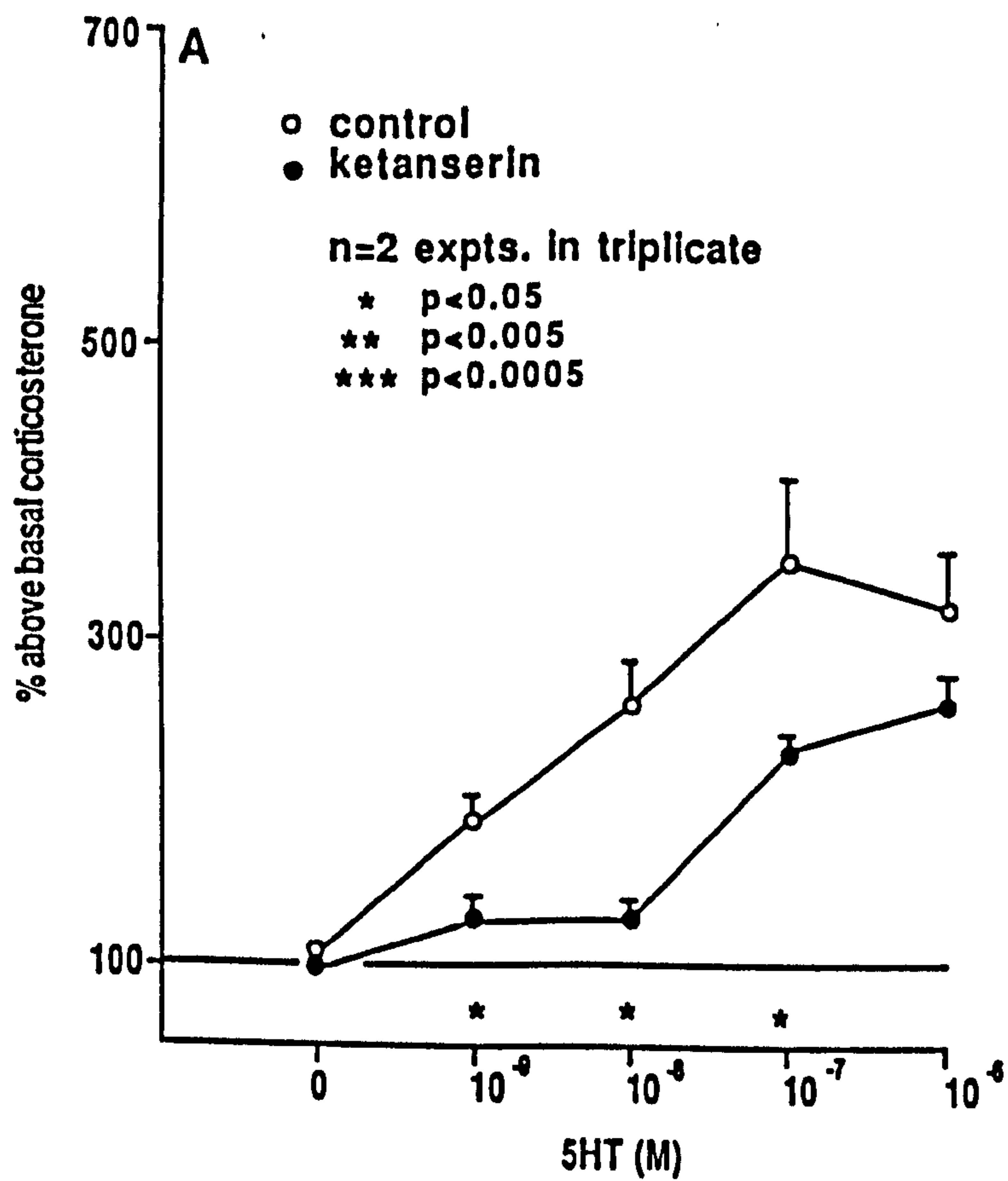


Fig 7.15 Effect of 10^{-6} M ketanserin on corticosterone and aldosterone output with 5HT and A II stimulated glomerulosa cells

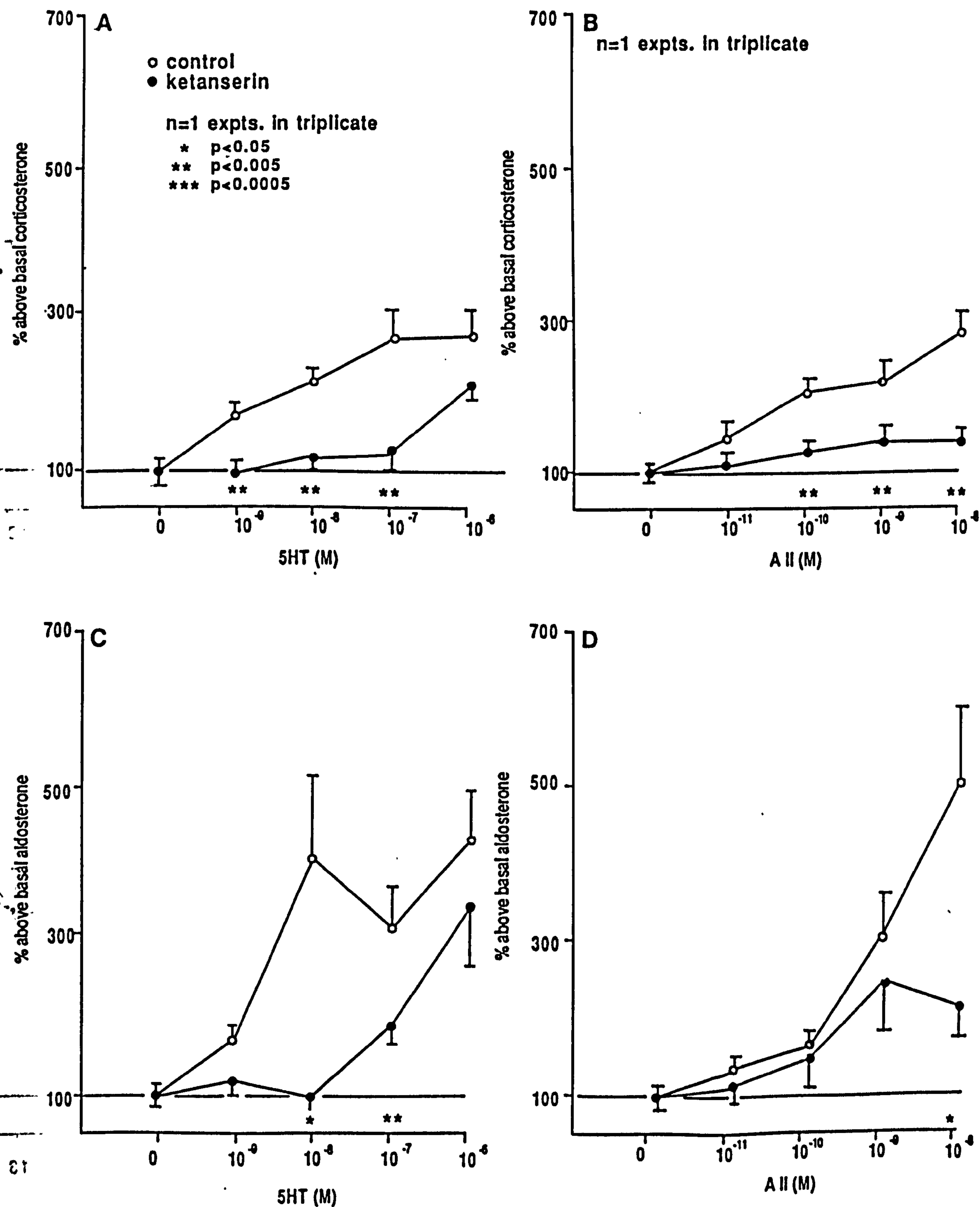
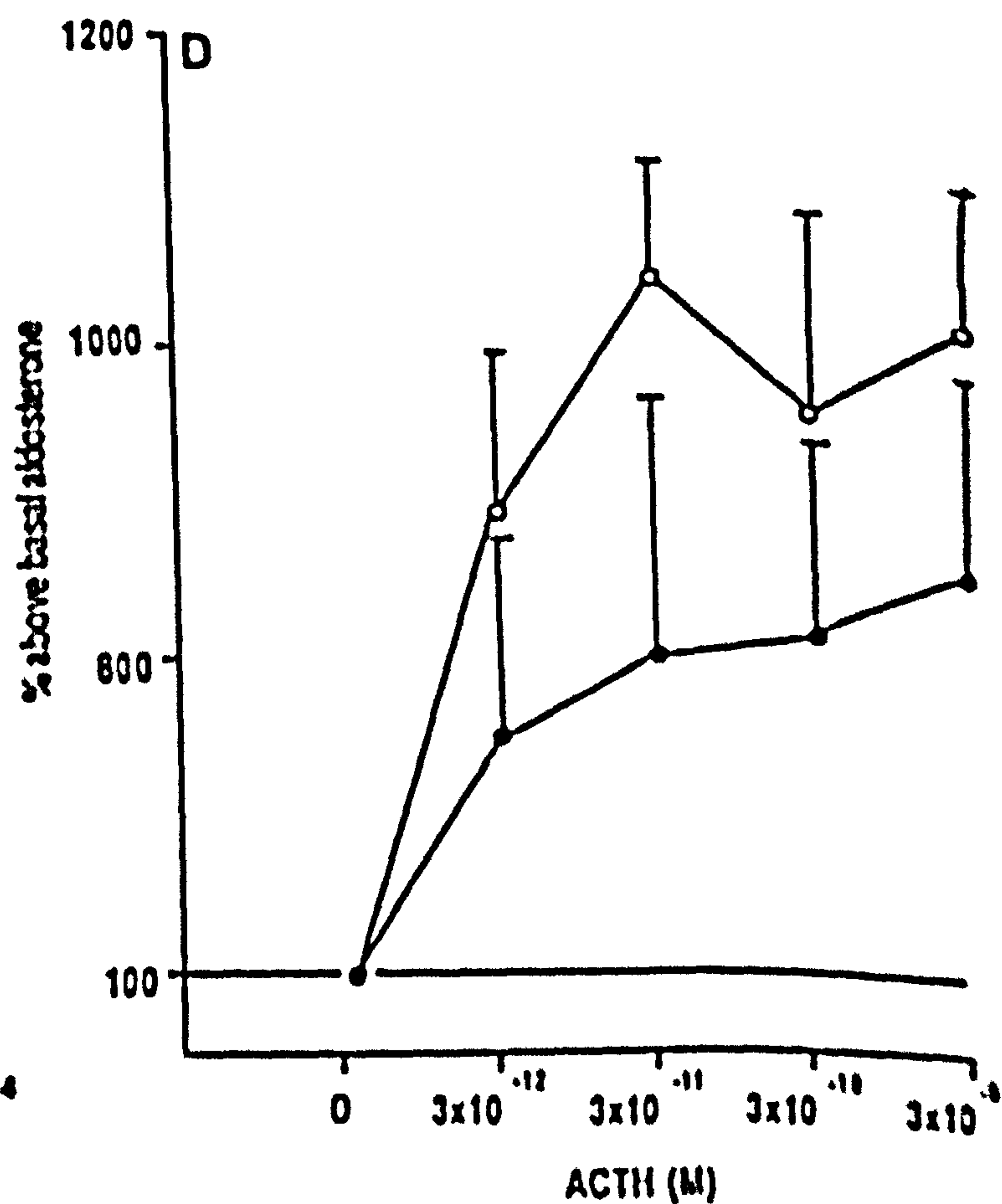
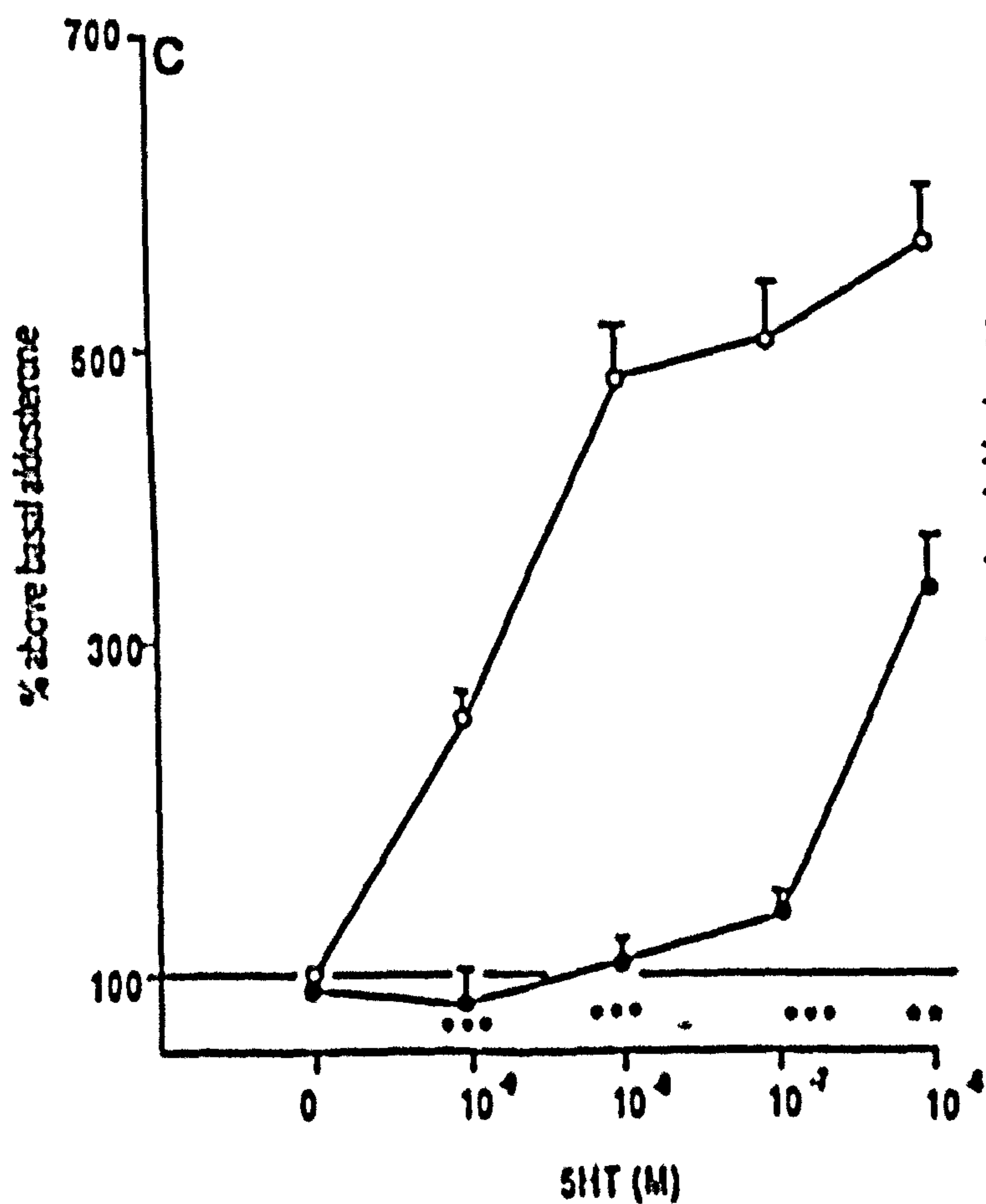
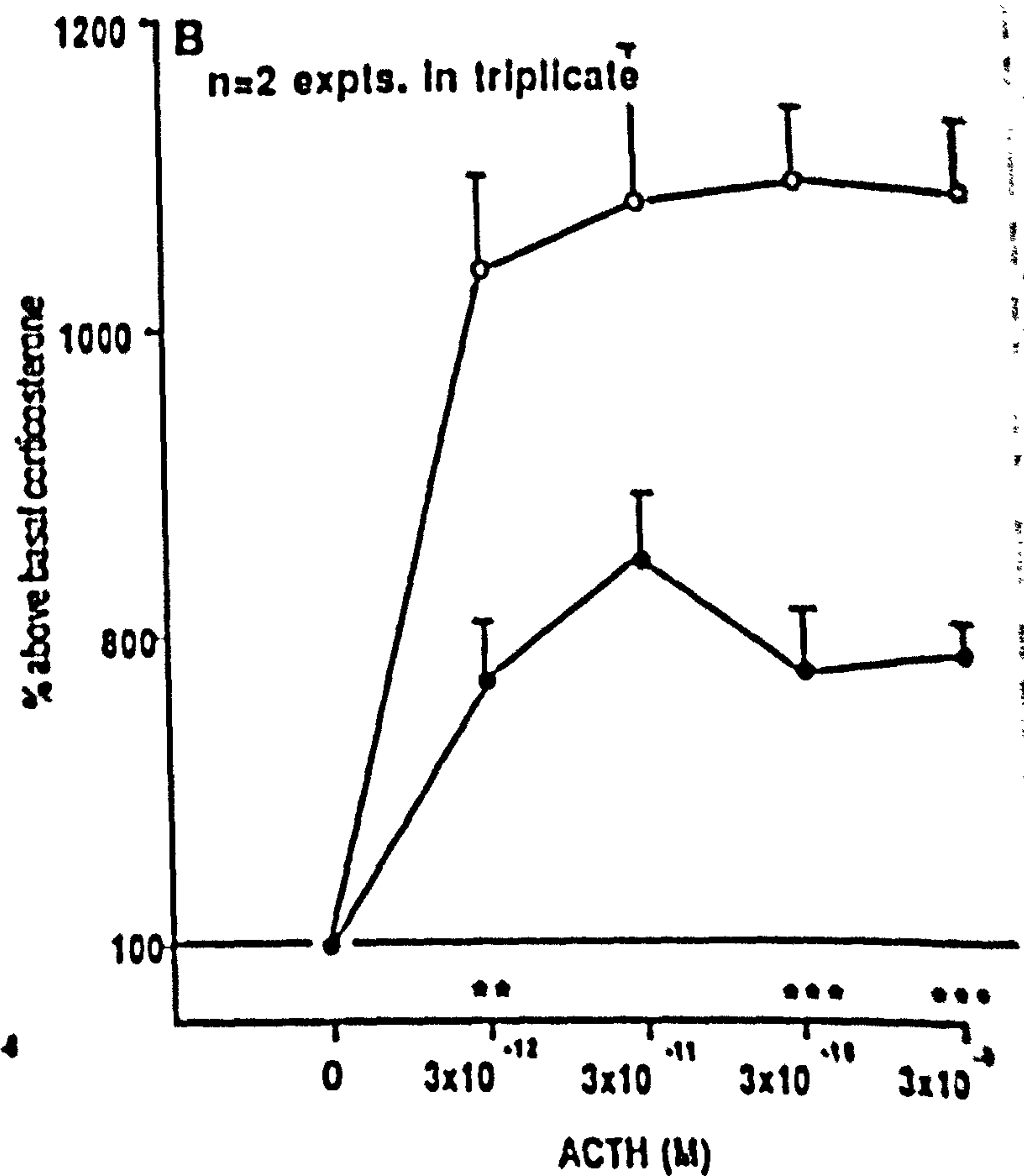
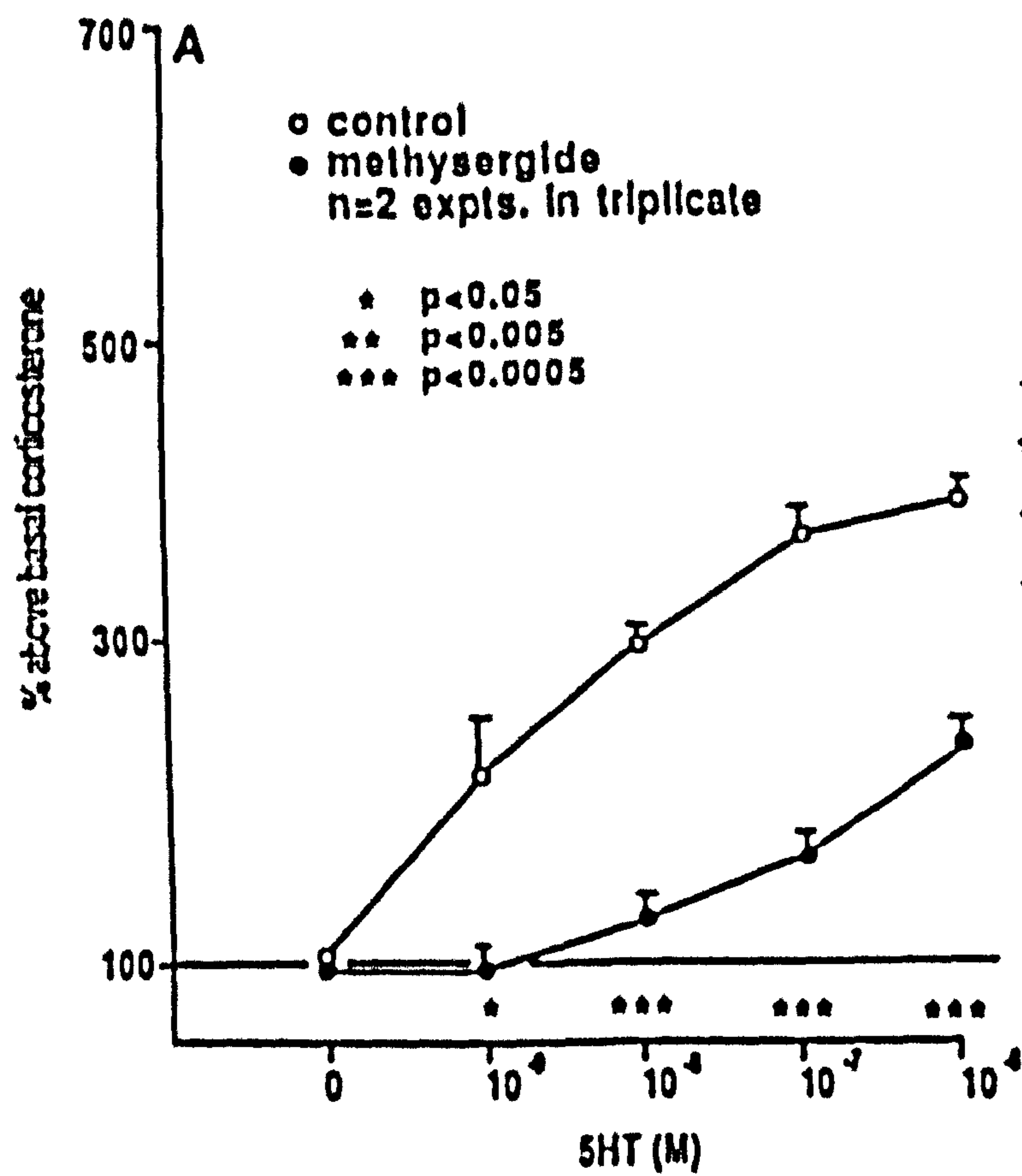


Fig 7.16 Effect of 10^{-6} M methysergide on corticosterone and aldosterone output with 5HT and ACTH stimulated glomerulosa cells



7.6.5 Effects of Methysergide on the Dose Response Curve to ACTH

Figure 7.16 shows the pooled data from two experiments for ACTH and 5HT stimulated steroidogenesis. At all doses of 5HT, methysergide significantly inhibited both corticosterone and aldosterone output. For ACTH, corticosterone was significantly inhibited at $3 \times 10^{-12} \text{M}$ ACTH, $3 \times 10^{-10} \text{M}$ and $3 \times 10^{-9} \text{M}$, although the percentage inhibitory effect at the highest dose of ACTH was not as great as for 5HT at 10^{-6}M (ACTH stimulated corticosterone was reduced by approximately 33% compared to 50% for 10^{-6}M 5HT). The aldosterone response was not significantly inhibited although the levels of significance and the large SEMs for each dose of ACTH may have disguised the inhibition as the graph clearly shows a tendency towards inhibition.

7.6.6 Methysergide in K^+ -stimulated steroidogenesis

Figure 7.17 shows the typical dose response curve to K^+ in M199 (0.2%BSA). Fakunding (1979) had shown the response to K^+ as plateauing at $8 \text{mM} \text{K}^+$ and upwards. Individual experiments appeared to show this, although pooled data for the aldosterone response curve was more in keeping with the biphasic response curve described by the Taits (1980a and b). At all doses, methysergide (10^{-6}M) did not affect K^+ stimulated steroid output whereas 5HT-stimulated steroidogenesis was inhibited at all doses with methysergide.

7.6.7 Effects of Methysergide on AII-stimulated Steroidogenesis

In a typical experiment, methysergide significantly inhibited aldosterone output in AII-stimulated cells at the higher doses of

Fig 7.17 Effect of 10^{-6} M methysergide on corticosterone and aldosterone output with 5HT and K^{\pm} stimulated glomerulosa cells

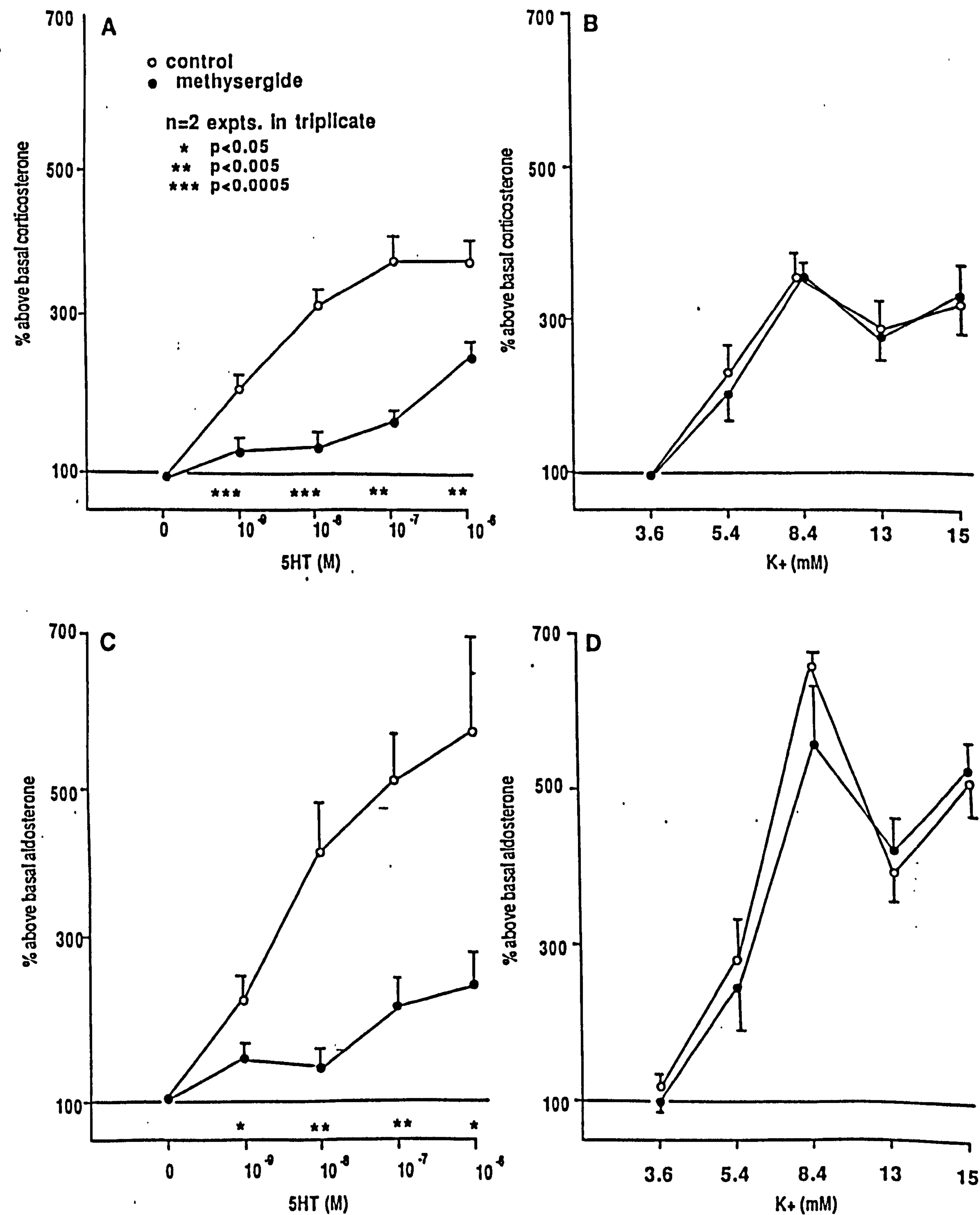
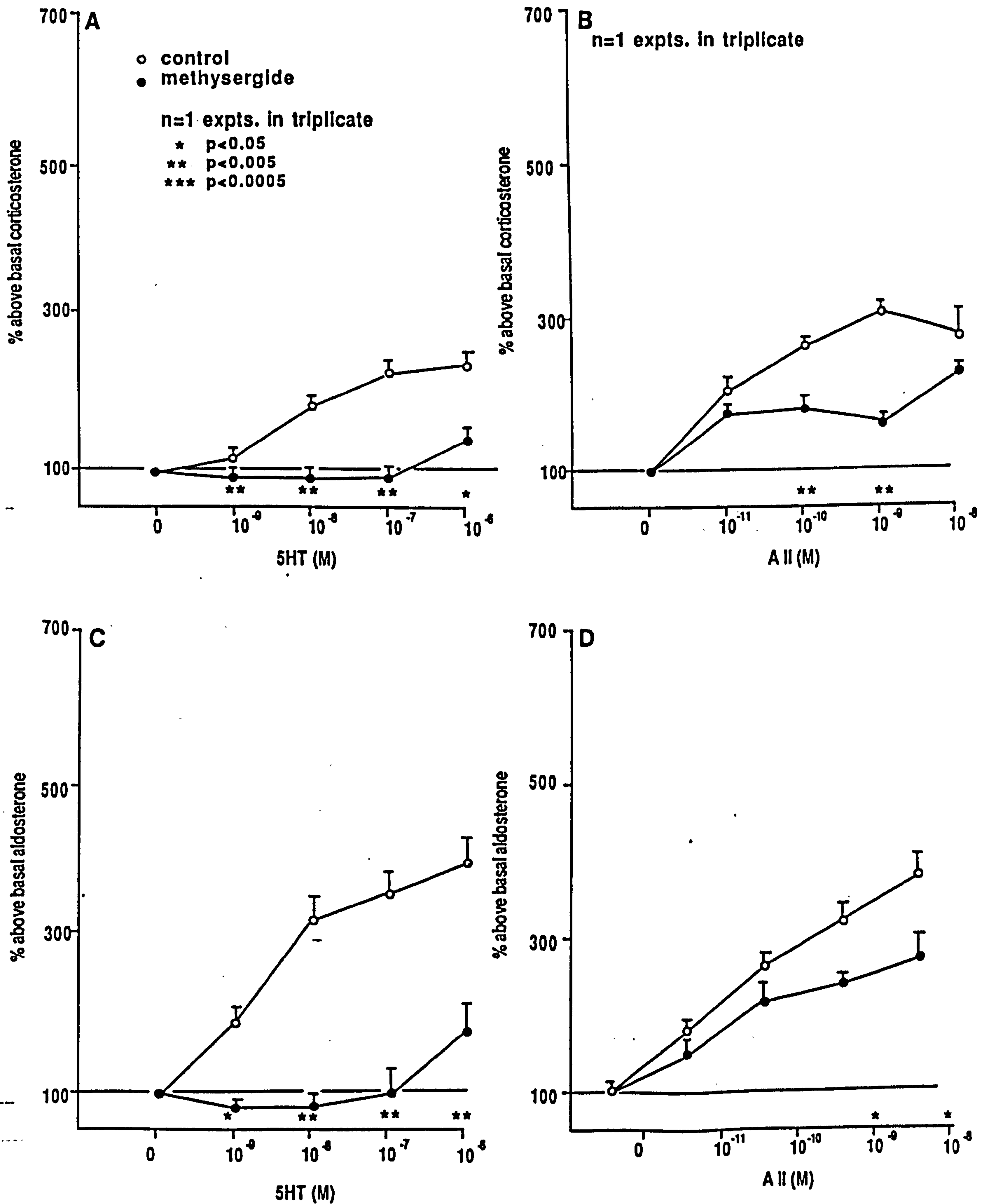


Fig 7.18 Effect of 10^{-6} M methysergide on corticosterone and aldosterone output with 5HT and A II stimulated glomerulosa cells



10^{-9}M and 10^{-8}M AII (Fig.7.18); corticosterone output was inhibited at the lower doses of AII (10^{-10}M - 10^{-9}M) and 5HT-stimulated steroidogenesis was significantly inhibited at all doses.

7.6.8 DISCUSSION

Methysergide in vitro inhibited 5HT stimulated steroid production to almost basal levels in all the experiments. The effects did not appear to be associated with a dose dependent inhibition as the 5HT concentration was increased. In Shah's (1979) study using rat renal cortical tissue stimulated with 10^{-4}M 5HT, elevated cAMP levels were inhibited by methysergide. However a very high dose (10^{-4}M) of antagonist was used and inhibition of cAMP may have been a result of interaction with dopamine receptors linked to the inhibition of adenylate cyclase i.e. D2 receptors (Missale et al., 1986) since dopamine receptors have been studied, in for example, the rat kidney (Felder 1984). Methysergide like several ergot derivatives, has dopaminergic agonistic properties, particularly at higher concentrations (Muller 1977). In an in vivo study undertaken by Krulich et al., (1978), infusions of 10mg/kg of methysergide (i.p.) into rats, inhibited the release of prolactin and could be overcome by pretreatment with a dopamine antagonist; Krulich (1981), proposed that at high doses, i.e. greater than 0.1mg/kg methysergide, the drug was acting as a dopamine agonist, whereas at lower doses it showed serotonergic antagonistic properties. Similarly the antagonist inhibited ACTH and AII stimulated steroidogenesis, although K^+

stimulated aldosterone secretion was not affected. This is in contrast to Mendelsohn's study with methysergide in which the drug inhibited K^+ stimulated aldosterone output at $5mMK^+$ (Mendelsohn 1981). The lack of inhibition in K^+ stimulated cells would argue against the notion that methysergide at $10^{-6}M$ has a toxic effect on the steroid secretions of the zona glomerulosa. Although dopamine has been associated with a tonic influence on basal aldosterone output (Carey et al., 1982), methysergide if it does exhibit dopaminergic properties in these experiments, does not significantly inhibit basal aldosterone secretion as Fig. 7.2 shows. However several groups have reported that dopamine inhibits AII stimulated aldosterone secretion both in vivo, and in vitro in rat, cow and human studies (McKenna et al., 1979., Birkhauser et al., 1979., Edwards et al., 1975, 1980a., Aguilera et al., 1984), so that methysergide's dopamimetic properties could be responsible for affecting the AII stimulated secretions. Carey (1982) had suggested that in vivo in man, dopaminergic inhibition of aldosterone was independent of AII and ACTH. Serotonin and dopamine are reported to have close interactions in several systems including brain striatal regions, which have close links (Lamberts et al., 1978); in other studies carried out with the dopamine antagonist metoclopropamide, in perfused rat adrenal cell columns, Edwards (1980a) , indicated that the potentiation of AII stimulated steroidogenesis could be a result of its serotonergic agonistic properties and suggested that the two receptors might be closely coupled. In another in vitro study

using isolated rat adrenals in a static system Lauer (1982) tested the effects of metoclopramide on AII, ACTH and K^+ stimulated steroidogenesis. In this study a dose of MCP as low as $3 \times 10^{-10} M$, significantly inhibited basal aldosterone secretion, which was ruled out as being a toxic effect. Further, the drug inhibited AII stimulated steroid output in a dose dependent way over an AII concentration of $2.4 \times 10^{-10} M$ - $2.4 \times 10^{-8} M$; at $3.5 \times 10^{-11} M$ ACTH aldosterone output was significantly inhibited although corticosterone was not, nor was the corticosterone output affected by MCP in ACTH stimulated fasciculata cells; $5.9 mM$ K^+ stimulated steroid output was also significantly inhibited. Lauer argued that MCP in this in vitro system, is acting at both 5HT and dopamine receptors, and its dopaminergic versus serotonergic antagonistic properties, are determined by the the relative concentrations of the amines within the tissue. To substantiate this hypothesis, incubating the cells under the same conditions with exogenous dopamine and MCP at increasing doses, produced an increase in aldosterone secretion in keeping with its dopamine antagonistic properties , so that assuming the cells have greater amounts of 5HT than dopamine, the antagonism to 5HT predominates. Despite the difficulty in understanding this concept in relation to drug antagonism, by a similar hypothesis, methysergide may be acting as a dopamine agonist and thus be responsible for the AII and ACTH inhibitory effects. The lack of effect on K^+ may be peculiar to this drug. In a similar in vitro study coordinated by Campbells group in 1981, increasing doses of MCP

(10^{-10}M - 10^{-5}M) were coincubated with AII, ACTH, K^+ and 5HT. Unlike Lauer's group who used a MCP concentration of $3 \times 10^{-4}\text{M}$ MCP, Campbell argued that this concentration and higher, had inhibitory effects on basal aldosterone and corticosterone output. Thus, they used a dose of 10^{-8}M - 10^{-5}M and found an inhibitory effect on AII stimulated steroid secretion; there was a slight inhibition of ACTH stimulated steroidogenesis and no effect on K^+ stimulated steroidogenesis. This is more in keeping with the results found here with methysergide, although in Campbell's work dose response curves to AII and ACTH in the presence of MCP, were not shown. However dose response curves to 5HT with two doses of MCP, 10^{-6}M - 10^{-5}M , showed competitive inhibition characteristics which they interpreted as MCP's serotonergic antagonistic properties. The interpretation was based on diagrammatical representation of the competition curves and analysis using the Schild plot method. This cannot be accurately determined using two antagonistic concentrations alone, and is therefore not a convincing interpretation of MCP's apparent competitive antiserotonergic properties; indeed, the fact that MCP also inhibits the AII and ACTH response curves, would either suggest that the drug is not a 'clean' drug for interpreting receptor effects or that both 5HT and/or dopamine, have close interactions with receptors for the other adrenal zona glomerulosa stimuli and in this regard, methysergide may also be included in this effect. It has already been indicated in a previous chapter that dopamine and 5HT modulate the response of basal and AII stimulated

steroidogenesis, moreover, dopamine has been found in relatively high concentrations within rat and bovine cortical tissue (Hannah et al., 1984., Racz et al., 1984) and concentrations of 5HT within the adrenal have been measured in appreciable quantities as shown in Chapter 8.

In in vivo experiments, Birkhauser (1979) showed that bromocriptine, a dopamine agonist with antiserotonergic properties also, modulated the plasma aldosterone response to infusions of AII and ACTH and Edwards (1975) showed that bromocriptine reduced the aldosterone response to furosemide in man, suggesting a dopaminergic inhibition; Sowers et al., 1980 showed that in vivo infusions of MCP directly stimulated plasma aldosterone secretion which was independent of PRA, ACTH and this was considered to be due to its anti-dopaminergic properties at the level of the adrenal cortex; Maestri et al., 1985 recently showed that MCP in vivo increased prolactin and plasma aldosterone levels independent of PRA and ACTH. However they argued that in this case, MCP was exerting a serotonergic stimulatory effect as well as an antidopaminergic effect, and infusions of pizotifen, a 5HT antagonist, reduced the plasma aldosterone increase to MCP, without affecting plasma prolactin levels in response to MCP. Methysergide, like MCP is not a 'clean' drug and like all ergot derivatives, has dopaminergic effects at high doses (Muller et al., 1977) and can displace binding of [³H]-dopamine and [³H]-spiroperidol from calf striatal membrane binding sites (Creese et al., 1976). However the in vitro and in vivo effects are complicated and

sometimes conflicting, but tend to suggest that there are close interactions between serotonergic and dopaminergic mechanisms in the control of aldosterone.

That ketanserin inhibited the 5HT- and AII-stimulated steroid response without affecting basal concentrations at 10^{-6} M, is more in keeping with a serotonergic interaction with AII stimulated steroidogenesis. Leysen (1981), had shown in brain tissue that ketanserin has a very low potency for dopamine receptors i.e. 100 times less than for 5HT₂ receptors so the likelihood of dopaminergic interactions is remote in this instance. Hoyer (1985) described [³H]-5HT recognition sites into subclasses 5HT_{1a}, 5HT_{1b} and 5HT_{1c}. [³H]-5HT has complex binding to all 5HT₁ receptors but they are distinguished by their ability to displace neuroleptics, agonists and β -blockers. In the case of 5HT_{1a} sites, these have high affinity for the neuroleptic spiroperidol and 8-hydroxy-2 (di-n-propylamino) tetralin (8OH-DPAT), and are in high concentration in rat and pig brain cortex and also have very low affinity for the 5HT antagonists methysergide, mesulergine and ketanserin. Recently however, these recognition sites have been correlated with adenylate cyclase activity (Kalkman 1985 cited in Hoyer's study 1985). 5HT_{1b} recognition sites have low affinity for spiroperidol, the new indole agonist RU 24969, and selectively bind β -blockers such as (-) [125] iodocyanopindolol [¹²⁵I] CYP in the presence of 30 μ M isoprenaline; they are now thought to be the subtypes of recognition sites that Fozard (1983a) described responsible for hyperlocomotory function in

rats treated with 5HT. The 5HT_{1b} recognition site also has low affinity for ketanserin and is thought to be functionally related to the 5HT autoreceptor described in canine saphenous veins, which inhibits the release of [³H]-epinephrine after electrical stimulation. 5HT_{1c} sites have high affinity for 5HT antagonists including pizotifen and [³H]-mesulergine; ketanserin shows relatively weak potency compared to its antagonism on 5HT₂ receptors. The overall data from correlating binding affinities of the agonists and antagonists to labelled 5HT recognition subtypes, tends to suggest that for example, 5HT which in these steroid experiments stimulates steroid production at approximately $2 - 5 \times 10^{-9}$ M 5HT, corresponds to a pK_d figure of between 8 - 9. Similarly, the pK_d values for significantly inhibiting 5HT stimulated steroidogenesis of approximately 6, for both methysergide and ketanserin, would correspond with values for displacement binding to 5HT_{1a} recognition sites (see Hoyer et al., 1985). 5HT_{1a} sites are thought to be associated with adenylate cyclase activation, which would be in keeping with the stimulation of cAMP in the steroid experiments. However, the ED₅₀ for stimulation of cAMP, approximated between 10^{-8} M - 10^{-7} M 5HT, which is an order of magnitude greater than for 5HT stimulated steroidogenesis. Ketanserin, although only weakly antagonistic, was more inhibitory at the lower doses of 5HT, around 10^{-9} M - 10^{-8} M, at levels at which cAMP was infrequently or not at all increased. Therefore the possibility exists that ketanserin is interacting with a subclass of 5HT_{1c} recognition sites, which may

also be involved in stimulated steroidogenesis and also respond to 5HT agonists, as seen in the indoleamine studies (Chapter 9). In Maestri's in vivo study (1985), inhibition of plasma aldosterone increase with pizotifen, could be due to interaction with 5HT_{1c} recognition sites at the adrenal cortex but this is still highly speculative. In the rat fundus, 5HT agonists stimulate contraction and bind to 5HT_{1c} recognition sites. The second messenger involved in recognition site coupling for the subclasses type 1b and 1c, have not been elucidated but are apparently dissociated from adenylate cyclase action; since type 2 receptor mediated responses have already been extensively associated with several messengers including prostacylin, calcium and/or PI (see Serotonin Receptors, Section 5.4, Chapter 5), it is conceivable that 5HT_{1c} receptors may also share similar second messenger modes of stimulation. The investigations are limited and further studies using a series of agonists displaced with an antagonist, in a physiological measured response system, as well as displacement studies for determining binding affinities of agonists and antagonists in the adrenal compared with brain regions, which have already been characterised for their recognition sites, need to be done before 5HT receptors (if they exist in the cortex) can be fully characterised in relation to the dose related response curve, consistently obtained in vitro in the rat zona glomerulosa.

Chapter 8

SEROTONIN IN THE ADRENAL GLAND

8.1

SEROTONIN IN THE ADRENAL GLAND

The origin of serotonin in the adrenal gland is not known. There exists the possibility that it is due to platelet release and is thus blood-borne (Osima et al., 1983). In the experiments and results described below adrenal 5HT was measured by HPLC before and after perfusion of the rat adrenal in situ with saline and simultaneous serum 5HT levels were also measured.

8.2 PREPARATION OF ADRENALS FOR 5HT DETERMINATION BY HPLC

Female Wistar rats of approximately the same age (8 weeks) and weight (100-120g) were ether anaesthetised and all the experiments were performed at the same time of day (9-10.30 a.m.) to reduce any possible diurnal variation in 5HT levels (Albrecht et al., 1956). Perfusion was performed by prior ligation of the ascending aorta and below the renal artery and vein bifurcation in the descending aorta. A small clip was placed anterior to a fine tube inserted into the renal artery to prevent blood flow from the aorta into the kidney and adrenal. The tube was held in place by a tight thread and connected to a 100ml plastic syringe containing ice cold saline which was gently pushed out into the renal and adrenal vasculature. Upon perfusion and within 2-5 minutes the adrenal and kidney became blanched, and the adrenals were rapidly removed and placed in plastic sealed tubes on dry ice before being stored at -70°C if they were not assayed on the same day. In the control experiments rats were anaesthetised and the adrenals were removed and assayed in the same

Comparison of Adrenal Serotonin Content in
Perfused v Non-perfused Adrenals (mean \pm SD \pm SEM)

Table 8.1

Perfused Capsular Adrenal tissue	[5HT] $\mu\text{g/g}$ wet wt.	[5HIAA] $\mu\text{g/g}$ wet wt.
	0.3 0.54 0.35 (3)	0.18 0.07 0.14 (3)
	0.39 \pm 0.1 \pm 0.07	0.13 \pm 0.045 \pm 0.03

() in parenthesis is number of experiments

Non-perfused Capsular Adrenal tissue	[5HT] $\mu\text{g/g}$ wet wt.	[5HIAA] $\mu\text{g/g}$ wet wt.
	0.3 0.5 0.35 (3)	0.07 0.04 0.07 (3)
	0.38 \pm 0.08 \pm 0.06	0.06 \pm 0.014 \pm 0.01

Statistics with Students paired t - test - no significant difference in perfused vs non-perfused capsular tissue.

way. Before homogenisation, adrenals were placed on ice and capsular portions separated from the rest of the adrenal to assay both portions separately.

The preparation and assay of 5HT for adrenals and whole blood were as described in chapter 5.

8.3 RESULTS

8.3.1 Capsular Region

Serotonin and its metabolite, 5HIAA were measured in the capsular portions as shown in Table 8.1. 5HT levels averaged $0.39 \pm 0.1 \pm 0.07$ (S.D., S.E.M.) $\mu\text{g/g}$ wet weight in perfused and non-perfused adrenals and 5HIAA levels were slightly but not significantly higher in the perfused capsules at $0.13 \mu\text{g/g}$ wet weight compared to $0.06 \mu\text{g/g}$ wet weight in the non-perfused capsules.

8.3.2 Decapsulated Adrenal

Table 8.2 shows 5HT levels in the non-perfused decapsulated adrenals at $2.24 \pm 0.9 \mu\text{g/g}$ wet weight compared to levels of $1.67 \pm 0.24 \mu\text{g/g}$ wet weight in the perfused decapsulated portions after perfusion, which was not significantly different. Similarly, 5HIAA levels (Table 8.2) did not change significantly.

8.3.3 5HT Serum Levels

Serum 5HT levels were measured and are shown in Table 8.3. The values averaged 4nmol/ml which is approximately equivalent to $0.7 \mu\text{g/g}$ wet weight.

5HT Concentration In Decapsular Adrenals : Perfused v Non-perfused
(mean ± SD ± SEM)

Table 8.2

Perfused Decapsular Adrenals	[5HT] µg/g wet wt.	[5HIAA] µg/g wet wt.
	1.6	0.06
	1.43	0.08
	2.0	-
	1.67 ± 0.24 ± 0.17	0.07 ± 0.01
Non-perfused Decapsulated Adrenals	[5HT] µg/g wet wt.	[5HIAA] µg/g wet wt.
	2.25	0.06
	1.13	0.06
	3.35	-
	2.24 ± 0.9 ± 0.64	0.06

Perfused adrenal 5HT content decreased by approximately 25%
(by Students unpaired t-test, this was not significant).

Serum 5HT Levels

Table 8.3

<div>-</div> <div>Rat</div>	{5HT} μmol/l
1	8.01
2	2.89
3	4.83
4	3.71
5	5.20
6	1.98
Mean (± SD ± SEM)	4.43 ± 1.93 ± 0.87

8.4 DISCUSSION

The results above suggest that most of the 5HT resides within the decapsulated portion of the adrenal gland. Unfortunately, it was not possible to further localise this to cortical as opposed to medullary regions. In perfusing the adrenals one might expect that removal of blood would reduce adrenal 5HT content. Although there was a hint of this, 5HT levels within the decapsulated adrenal were still considerably higher per unit wet weight than that measured per ml or per gram in whole blood in which platelets had been disrupted to release all bound stores of 5HT. Capsular tissue contained only approximately 25% of the concentration of 5HT in the inner adrenal and closely matched blood levels, which may suggest that disruption of contaminating platelets during homogenisation could have been responsible for the capsular levels measured. However, prior perfusion did not reduce the concentrations measured which would tend to indicate that significant levels of 5HT are present in the capsular regions of the adrenal cortex. As discussed in Chapter 9, it would appear that exogenous 5HT can be stored in the inner cortical regions.

After the early separation and extraction methods for identifying 5HT pioneered by Vanable et al., 1963, it became possible to measure 5HT in organs previously not thought to contain any, and Snyder (1965) detected 5HT in the adrenal gland at 0.45 µg/g wet weight levels. At around this time Potter and Axelrod (1963) localised 5HT, after exogenous administration of [¹⁴C]-5HT, in the chromaffin granules,

using sucrose differential centrifugation. More recent methods using HPLC for measuring small amounts of tissue 5HT, have estimated levels of 1.4 - 1.5 $\mu\text{g/g}$ wet weight in the whole adrenal (Kuriyama et al., 1984., Verhofstadt et al., 1983). Further, it has been proposed by Kuriyama's group that this endogenous 5HT is located in the adrenaline containing chromaffin granules rather than the noradrenaline containing granules. Exogenous administered 5HT by Bertler et al., in 1960, into rats showed that this too was taken up by the chromaffin granules. Biochemical determinations correlated with immunohistochemical staining studies by Holzwarth (1983) and Verhofstadt (1983), within the medullary region of the rat, although Petrovic (1984) could not localise it by histochemistry in this species (see also Chapter 11). Blood levels of 5HT were determined in dogs by Humphreys as early as 1954, who estimated values of $0.4 \mu\text{g}/10^9$ of 5HT in platelets, whilst Erspamer reported 5HT levels in rat serum of $0.57 \mu\text{g}/\text{ml}$, which closely parallels these determinations. So far then, it is more than probable that 5HT is an endogenous amine in rat adrenals, but where this arises, i.e. from blood, and is taken up into chromaffin granules and stored, or taken up into catecholaminergic nerve endings, thus displacing endogenous catecholamine stores present in the adrenal cortex (Kleitman et al., 1985 see Nerve Innervation section) or is locally present within contaminating mast cells, which in the rat and mouse store and take up 5HT from the circulation, to be released during physiologically controlled mechanisms, is not known.

8.5 MANIPULATION OF ENDOGENOUS 5HT LEVELS

One way of looking at the physiological importance of 5HT in the control of aldosterone secretion at the level of the adrenal cortex, is to alter the endogenous levels. Serotonin levels can be increased by the administration of precursors such as tryptophan and 5-hydroxytryptophan in vivo, or can be decreased by drugs that release endogenous stores, or alternatively by administration of drugs that inhibit 5HT biosynthesis. In a series of studies blood levels of 5HT in rats, were depleted or elevated by administration of pharmacological agents, and the in vitro response to 5HT and AII was analysed. Angiotensin II was looked at in the first instance because 5HT antagonists were shown to be potent inhibitors of the aldosterone response to AII.

8.5.1 PCPA Administration

Parachlorophenylalanine (PCPA) is a neutral amino acid derivative that in vivo is a non-competitive inhibitor of tryptophan hydroxylase. Koe (1966) showed that a single dose of PCPA in rats caused a marked and relatively selective depletion of 5HT in the brain (although it is also a potent inhibitor of peripheral 5HT biosynthesis). PCPA causes a relatively rapid depletion of 5HT within 24 hours which remains depressed for approximately one week (Fuxe et al., 1971). The disadvantages of PCPA include: (1) high doses of the drug have to be given which may affect tissue uptake and utilisation of other amino acids; and (2) PCPA also depletes

catecholamine. However, the time course for catecholamine depletion is longer when compared to 5HT depletion so that a short time study of PCPA's effects reduces the catecholamine depletion effects of the drug, and thus its involvement in any interpretation of results.

In preliminary experiments a time-course study of the effects of a single dose of PCPA (i.p.) on peripheral stores of 5HT, (in particular in the adrenal gland) were estimated in male Wistar rats 200-250g: serum 5HT levels were used as a marker of the change in 5HT synthesis since platelets take up 5HT from biosynthetic sites such as the GI tract.

8.5.2 Rat Cannulation and Infusion of PCPA (i.p.)

Male Wistar rats were used in this series of experiments since being larger, it was easier to perform cannulation operations. Rats between 200-250g were anaesthetised using ether and a cannula was placed in the carotid artery and exteriorised at the back of the neck.

After allowing a 48 hour recovery period, blood samples were collected on day 3 at 11.00 a.m. in all rats divided into control and PCPA/5HTP treated groups immediately before drug treatments, i.e. day 3 values were pre-drug control values for each set of rats, measuring plasma corticosterone (B), aldosterone, PRA and 5HT as described in Chapter 6. On day 4, 24 hours after the first drug administration, blood samples were taken from each set of rats, and immediately after, another dose of drug administered; on day 5 another 24 hours after the previous dose, and 48 hours after the first dose of drug, blood samples were again taken and the rats sacrificed by cervical

dislocation as described in Chapter 6. The steroidogenic responsiveness of cells obtained from PCPA/5HTP treated rats were monitored relative to control rats which were treated with saline, using AII and 5HT as stimuli.

8.5.3 5HTP Administration

5-Hydroxytryptophan is regularly used for increasing 5HT levels and is the substrate for the enzyme aromatic-L-amino acid decarboxylase. This enzyme is ubiquitous and is not restricted to 5HT synthesising sites. Thus, 5HTP can be converted to 5HT in catecholaminergic neurones containing the enzyme, and deplete catecholamine stores as a result of deposition of 5HT. To reduce the non-specific uptake of 5HTP into tissues, lower doses (20-100mg/kg) have been used (Osborne 1982) and in this study L-5HTP at a dose of 20mg/kg was administered (i.p.) into cannulated male rats.

8.5.4 5HTP Administration into Cannulated Rats

Rats were cannulated as described above; control rats were injected with saline (i.p.) on days 3 and 4 and 5HTP treated rats were injected at the same time with 20mg/kg of L-5HTP. Blood samples were collected at similar time intervals over 3 days as for the PCPA study, and on day 5 after blood samples were obtained, the rats were killed and adrenals isolated for in vitro experiments, measuring the aldosterone secretion in response to AII and 5HT.

**Serum 5HT Levels after Single Dose (i.p.)
of PCPA (320 mg/kg) - Time Study**

Table 8.4

Day	1	2 h	6	8
Control {5HT} μmol/l	4.60 3.40	5.00 3.15 3.40	1.50 4.65	2.20 1.20 2.02
Mean	4.00 ± 0.60	3.85 ± 0.80 ± 0.60	3.07 ± 1.57 ± 1.59	1.81 ± 0.43 ± 0.31
+ PCPA {5HT} μmol/l	1.00 3.00 1.54	1.90 1.43 2.70	0.87 3.40	4.20 2.80 4.35
Mean ± SD ± SEM	1.87 ± 1.04 ± 0.60	2.01 ± 0.60 ± 0.40	2.13 ± 1.80 ± 1.26	3.78 ± 0.80 ± 0.49

Table 8.5

**Whole Adrenal 5HT Levels after Single Dose
(i.p.) PCPA (320 mg/kg) Time Study**

Day	1	2	6	8
Control {5HT} μg/g wet wt. (whole adrenal)	0.79 1.30	0.67 0.54	0.88 1.20	13.00 10.56
Mean	1.04 ± 0.25	0.60 ± 0.06	1.04 ± 0.16	11.78 ± 1.22
+ PCPA {5HT} μg/g wet wt. (whole adrenal)	0.47 0.40	0.37 0.40	0.74 0.35	2.20 1.23
Mean ± SD	0.43 ± 0.05	0.38 ± 0.02	0.54 ± 0.27	1.75 ± 0.48

8.6 RESULTS

8.6.1 PCPA Time Study

Table 8.4 shows the concentration of 5HT in $\mu\text{mol/l}$ in rat serum over 8 days in control and PCPA-treated rats. On day 1 5HT levels approximated $4.0\mu\text{mol/l}$ in the control group compared to $1.87\mu\text{mol/l}$ in the PCPA group which showed a depressing trend although this did not reach a level of significance until day 2 ($p < 0.05$); on all following days 5HT levels in the PCPA and control groups were not significantly different.

Table 8.5 shows the corresponding changes in 5HT levels in the whole adrenal over the same time period in the same group of rats. Here again there was a trend towards reduction in 5HT levels 24 hours after a single dose of PCPA from $1.04\mu\text{g/}$ wet weight to $0.43\mu\text{g/}$ wet weight in the treated group although on this and day 2, the levels were not significantly different by statistical analysis. However, only a few rats were taken in this limited study and a greater number of measurements given more time might have delineated a clearer trend; there were also anomalies in the measurement of 5HT in the control adrenals compared to the PCPA treated rats on day 8. The levels were exceedingly high compared to the PCPA group and even in comparison to the other control data, levels of 5HT were very high.

8.6.2 DISCUSSION

As mentioned earlier, brain 5HT levels are reduced within 24 hours and remain depressed for up to a week after a single dose of PCPA; in these studies it appears that there is a trend towards a close parallel in 5HT levels in serum and adrenals at 24 hours and 48 hours, but 5HT levels return to control values more rapidly in the blood compared with the adrenal gland as indicated by percentage changes. For example, after day 6, 5HT levels in the serum in PCPA treated rats approached $2/3$ the control levels, compared with approximately $1/2$ 5HT concentration in the PCPA treated adrenals after day 6, whereas after day 1 and day 2, 5HT levels in both the adrenals and the blood respectively, approximated $1/2$ the control value. Thus, over 24 hours and more significantly at 48 hours, serum levels may be a good indicator of what is happening at the level of the adrenal after a single dose of PCPA. However, there may be differential recovery of several 5HT synthesising organs within the rat after PCPA administration, thus after a maximum of 48 hours, some organs, such as the GI tract, may begin to recover and synthesise 5HT which would be reflected as an increase in 5HT storage in platelets. In contrast, synthesis of 5HT within the adrenal appears to show slower recovery after PCPA administration. This data would argue for a possible local synthesis of 5HT within the adrenal as well as/or blood contaminating 5HT levels. The previous results showed that perfusing capsular and decapsulated adrenals, did not completely remove 5HT.

**PLASMA ALDOSTERONE AND CORTICOSTERONE VALUES
IN ± PCPA TREATED RATS**

Table 8.6 Plasma Aldosterone ng/100 mls

Days 3		4		5	
Control	+ PCPA	Control	+ PCPA	Control	+ PCPA
17.86	59.8	17.86	214.3	17.85	58.9
48.2	57.1	58.9	73.2	75.0	73.2
58.0	32.1	13.4	38.4	223.0	54.4
44.6	17.8	69.6	50.0	35.7	59.8
80.35	20.5	141.1	53.6	29.5	-
75.0	-	25.8	-	-	-
25.8	-	-	-	-	-
49.9 ± 21.6 ± 8.9	37.1 ± 17.3 ± 8.7	54.4 ± 44.0 ± 21.6	85.9 ± 65.0 ± 33.0	76.2 ± 75.8 ± 38.0	61.6 ± 6.9 ± 4.04
N.S.		N.S.		N.S.	

Plasma Corticosterone ng/ml

Table 8.7

Days 3 *		4 *		5	
Control	+ PCPA	Control	+ PCPA	Control	+ PCPA
307	418	96	825	58	135
289	446	638	563	536	1140
590	206	690	552	500	820
470	164	166	477	359	500
329	37	599	602	582	-
350	-	717	-	-	-
345	-	-	-	-	-
383 ± 100 ± 41	254 ± 78	484 ± 253 ± 114	603 ± 119 ± 60	407 ± 373 ± 215	649 ± 373 ± 215
N.S.		N.S.		N.S.	

Each value ≡ 1 rat

* p<0.05

8.7 IN VIVO EFFECTS OF PCPA AND 5HTP ON BLOOD PARAMETERS AND THE RESPONSIVENESS OF ZONA GLOMERULOSA CELLS TO AII AND 5HT

8.7.1 PCPA Treatment

Tables 8.6 and 8.7 show the results of one PCPA experiment, although two similar experiments showed similar trends; PRA values were incomplete for all days on individual experiments and are thus not included in the final analysis. Plasma corticosterone and aldosterone were measured on days 3, 4 and 5 and in all cases PCPA did not significantly alter the levels compared to control values or between the same group on different days except between day 3 and 4 for the PCPA rats ($p < 0.05$), where day 3 represents pre-drug plasma B values, and day 4, plasma B values 24 hours after the first dose of PCPA; plasma aldosterone was not significantly different in the same group.

However, over the same time scale 5HT levels (Fig.8.1) in the serum were significantly depressed ($p < 0.001$) by day 5 from $8.6\mu\text{mol/l}$ to $1.6\mu\text{mol/l}$ when the adrenals were used in in vitro studies.

8.7.2 Responsiveness of Zona Glomerulosa Cells to AII and 5HT in Control and PCPA Treated Rats

As can be seen in Fig.8.2, basal steroid output of aldosterone in control and PCPA-treated rats, were significantly different ($p < 0.05$), with depressed steroid output in the PCPA group compared to the control group. At all doses of stimulus, a steep dose response curve was achieved in the control group. In the PCPA group there is still

Fig 8.1

Whole serum serotonin levels in \pm PCPA treated rats

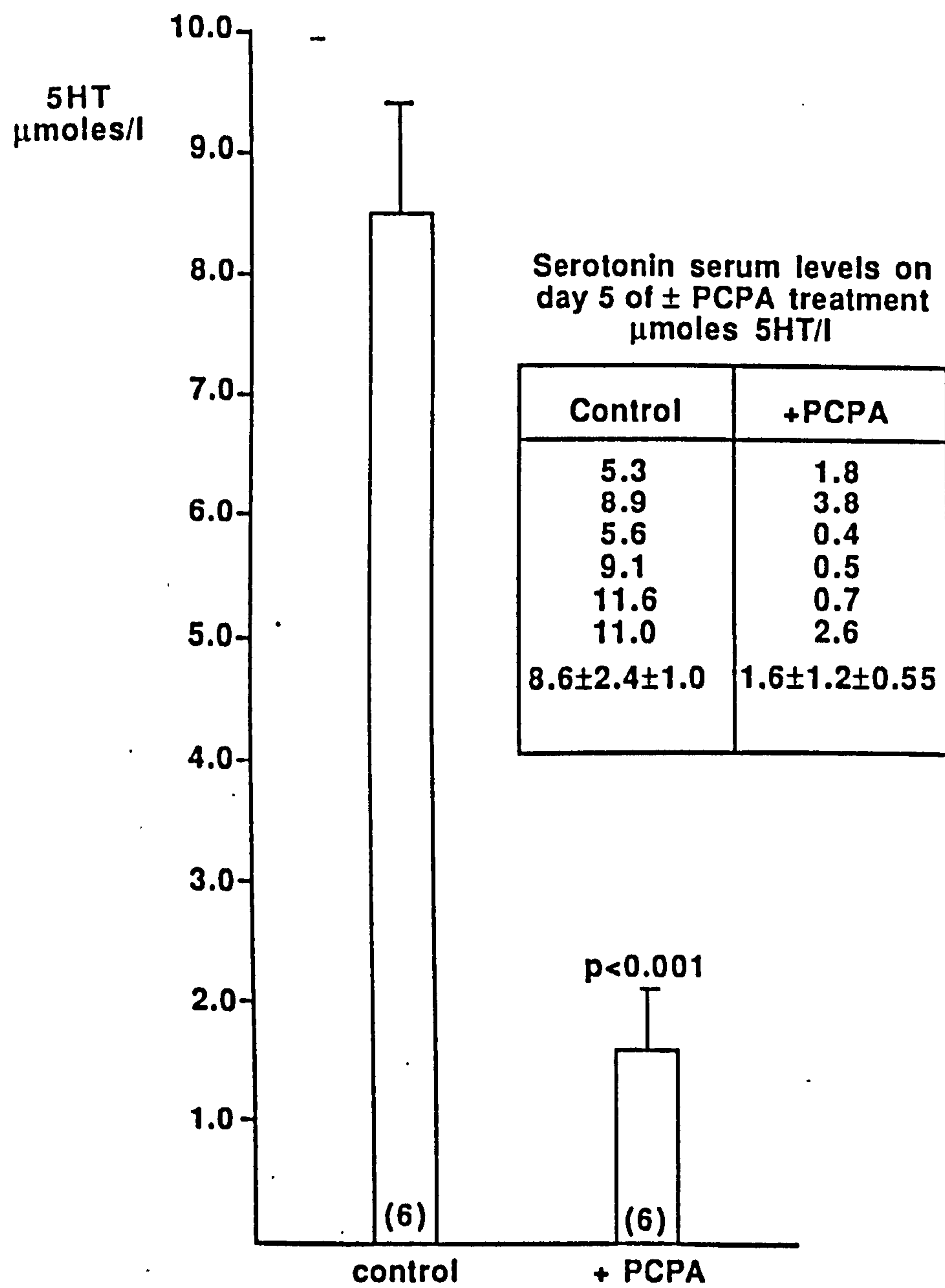
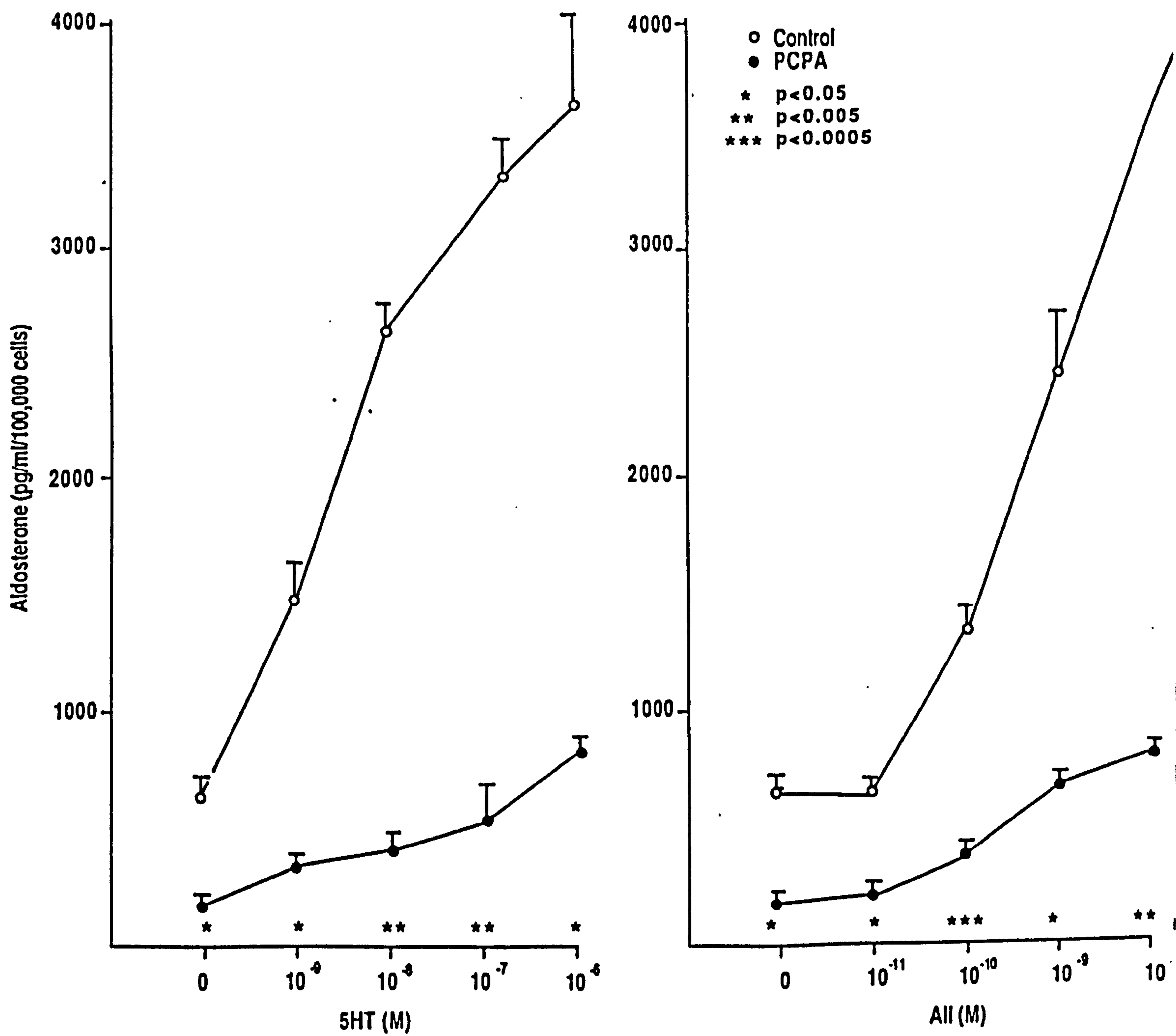


Fig 8.2

Dose response curve to 5HT and All in control vs PCPA treated rats in isolated zona glomerulosa cells



Plasma PRA, Aldosterone & Corticosterone Values In \pm 5HTP Treated Rats

PRA ng/ml/hr

Table 8.8

Days 3		4		5	
Control	+5HTP	Control	+5HTP	Control	+5HTP
60.2	41.6	100.48	21.2		32.0
60.2	41.6	34.0	30.0	28.0	7.8
40.4	20.1	96.0	22.0	40.0	34.8
40.4	80.4	24.0	1.6	52.0	-
-	20.6	41.2	-	24.0	-
-	70.9	10.6	-	-	-
62.0	47.6	52.3	25.1	31.0	24.8
± 12.4	± 6.22	± 35.0	± 5.7	± 14.0	± 12.0
± 7.4	± 8.8	± 14.1	± 3.3	± 7.0	± 8.6
N.S.		N.S.		N.S.	

Table 8.9

Aldosterone ng/100 mls

Days 3		4		5	
Control	+5HTP	Control	+5HTP	Control	+5HTP
17.8	38.4	80.35	60.7	48.2	58.9
47.2	25.0	33.9	21.4	23.2	33.9
19.6	214.0	46.4	20.5	80.3	26.8
-	18.75	25.0	-	77.7	-
-	116.0	-	-	-	-
28.2	82.4	46.4	34.2	57.3	39.8
± 13.5	± 74.0	± 21.0	± 18.7	± 23.4	± 13.7
± 9.5	± 37.0	± 12.0	± 13.0	± 13.5	± 9.7
N.S.		N.S.		N.S.	

Table 8.10

Plasma Corticosterone ng/ml

Days 3		4		5	
Control	+5HTP	Control	+5HTP	Control	+5HTP
430	612	182	560	91	248
599	230	412	220	485	353
858	647	248	99	248	509
103	83	128	232	-	-
-	696	299	-	-	-
-	504	-	-	-	-
462	495	277	254	370	259
± 249	± 316	± 197	± 110	± 131	± 147
± 102	± 158	± 99	± 49	± 76	± 65
N.S.		N.S.		N.S.	

a clear and significant dose response curve to each stimulus, however the absolute levels are significantly reduced at all doses compared to the control response. Although basal levels are depressed, the lowest dose for stimulating steroid output above basal levels, is not altered, i.e. the sensitivity by this definition, is not altered, but rather the responsiveness is reduced significantly at all doses.

8.8.1 5HTP Treatment

Tables 8.8, 8.9 and 8.10 show plasma PRA, corticosterone and aldosterone respectively in control and 5HTP-treated rats on days 3, 4 and 5. Over the time period of blood sampling no significant changes occurred in PRA, corticosterone or aldosterone in the 5HTP treated rats compared to the control group. However, 5HT levels were significantly elevated ($p < 0.05$) in the serum by day 5 when in vitro experiments were performed (see Fig. 8.3).

8.8.2 Responsiveness of Zona Glomerulosa Cells to AII and 5HT in Control and 5HTP Treated Rats

Figure 8.4 shows the dose response curves to AII and 5HT and aldosterone output per 100,000 cells in one experiment representative of two other experiments showing similar results. The basal steroid output was not increased in the 5HTP group in this experiment although in both other experiments basal levels were increased relative to the control group. However, in these two experiments, serum 5HT levels were not significantly elevated by day 5 compared to the control group. In the data shown (Fig. 8.4 and Fig. 8.3) 5HT

Fig 8.3
Whole serum serotonin levels \pm 5HTP treated rats

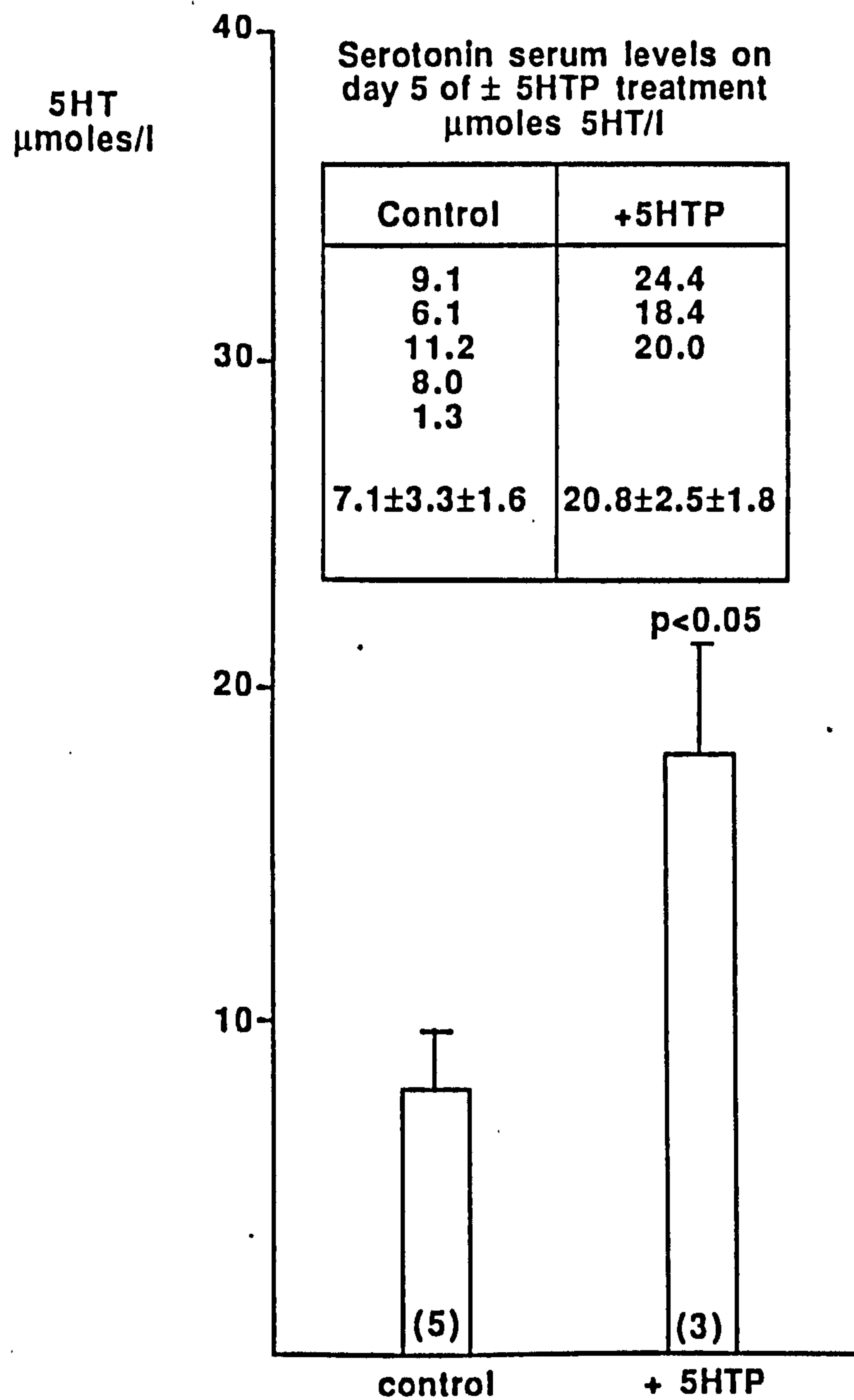
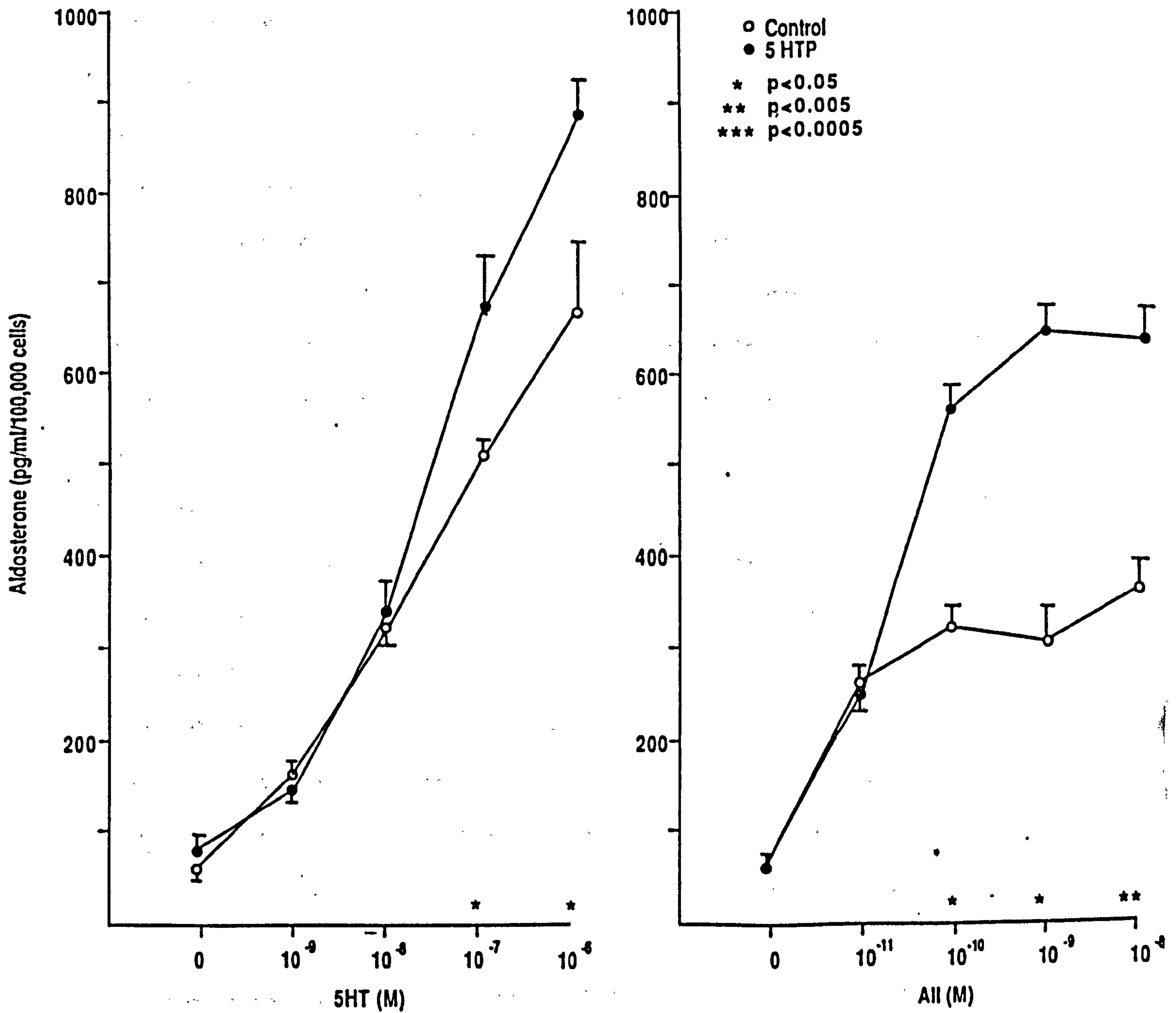


Fig 8.4

Dose response curve to 5HT and All in control vs 5 HTP treated rats in isolated zona glomerulosa cells



serum levels were increased by day 5, and the dose response curve was elevated at the higher doses of the stimuli, although the sensitivity of the response was not altered, the maximum responsiveness appeared to be increased by 5HTP treatment.

8.9 DISCUSSION

From the in vitro findings it would appear that depletion of 5HT in the whole animal causes a marked and reproducible depression of basal steroid output at the level of the adrenal. However in the case of the 5HTP experiments, although the responsiveness and sometimes basal steroid levels were increased by 5HTP treatment, serum 5HT levels did not always parallel these changes, thus changes in 5HT levels in the serum may be dissociated from the effects at the level of the adrenal.

Blood samples were taken at 24 hour intervals prior to a dose of PCPA on the same day, so that plasma steroid measurements recorded the effects of the previous dose of the drug 24 hours before. Thus, the chronic effects on these parameters indicated that 5HT depletion did not result in a reduction in plasma aldosterone levels in each group or relative to one another i.e. inter- and intra-groups, unlike the in vitro findings; interestingly after the first 24 hours of PCPA treatment, plasma corticosterone within the same group of rats on PCPA treatment, was elevated thus the drug may be acting on the higher centres, possibly affecting ACTH and other pituitary factors (see later) although the trend is in the opposite direction to the

work of Vernikos-Danellis (1973) and is not readily explicable. What is probably a more valid measure are the plasma values for each group of rats on a given day, and in this case, plasma parameters did not alter for any day. PCPA takes at least 24 hours to deplete 5HT levels so that measurements over this time period are valid in indicating an effect of 5HT depletion as opposed to the injection of a drug per se. The in vitro experiments are unique in that they isolate the zona glomerulosa cells from all circulating factors that together influence plasma aldosterone levels, so this allows one to look at the effects of individual stimuli. In this case, if 5HT depletion is having an effect on aldosterone output in vivo, the effects are masked by other factors which also regulate basal steroid output, such as AII (Aguilera et al 1978a and 1978b) in the rats, although pituitary factors especially in the sodium deplete state, are thought to exert an important regulatory influence (Palmore et al., 1970., Pratt et al., 1981, Brown et al., 1979., McCaa et al., 1974., Dawson-Hughes et al., 1981). It is interesting however, that increasing 5HT levels increases basal output in some experiments and decreasing 5HT levels has the opposite effect, in that the levels of the indoleamine appear to exert a prominent 'tonic' influence on steroid output, either within the periphery or at higher centres and further, this tonic influence also modulates the dose response to AII and 5HT in the in vitro set up.

This is similar to the in vitro and in vivo effects seen with dopamine, which has been regularly postulated as exerting a tonic

inhibitory influence both on basal and AII stimulated steroidogenesis (Carey et al., 1982., Edwards et al., 1975, 1980a., Noth et al., 1980., Aguilera et al., 1981,1984., Lauer et al., 1982., McKenna et al., 1979). Indeed Aguilera has postulated that dopamine exerts a potent inhibitory effect on the dose response to AII infusions in rats on high salt intake, which can be overcome with coinfusions of dopamine antagonist, metoclopropamide. In man, Drake et al., 1984, found that the plasma aldosterone response to AII infusions in low salt diet, was also blunted by the administration of dopamine, which more directly involved dopamine in modulating the response; the effects of dopamine could not be seen in normal sodium status. It is conceivable that the effects of dopamine are closely interactable with a serotonergic tonic influence in differing salt status, i.e. in the high salt state, 5HT may be reduced or may not change (see Chapter 10), but be effectively reduced in relation to dopamine concentrations; the converse may hold for the low salt state (see Chapter 10). In the results obtained in vivo for PRA in 5HTP-treated rats, the effects of 5HT on the adrenal appeared to be independent of a renal influence in the chronic measurement; Nisbet (1987) however, showed that acute measurements of PRA and plasma aldosterone, after 5HTP administration, revealed an increase in both parameters with increased 5HT serum levels. It has already been discovered that there are neural links between the hypothalamus and the kidney (Dempster et al., 1978) and 5HT within the brain has several putative functions as a neurotransmitter, both within the brain and also

possibly as a sympathetic neurotransmitter within the renal nerves. Petrovic showed that there existed large quantities of 5HT within the kidney although neural containment was not mentioned (Petrovic et al., 1984). However it has been shown by several groups that 5HT acts centrally to induce changes in PRA concentrations in the plasma. Zimmerman (1980), injected 5HTP into dogs to increase 5HT levels in the brain and periphery and by differential inhibition of the peripheral decarboxylase enzyme using carboxydopa, showed that PRA levels were significantly elevated 15 and 30 minutes after the injection of 5HTP whereas the PRA levels were not increased when brain decarboxylase was also inhibited with benserazide. These results do not rule out a peripheral effect of 5HT on PRA but rather suggest a more prominent role for 5HT within the brain controlling PRA, at least acutely. However, the lack of conclusive results for 5HT precursors and depletors on PRA in this chronic study are in keeping with other studies. Maestri (1985), showed that plasma aldosterone was increased without concomittant changes in PRA and potassium when MCP was administered and this could be reduced by administering the serotonin antagonist pizotifen, thus suggesting a tonic effect of 5HT on plasma aldosterone at least when measured in an acute study over an hour. Sowers et al., 1980 found similar effects with MCP in vivo but had postulated that this was a dopaminergic phenomenon. Similarly, direct infusions of 5HT into man suggested to Mantero and co-workers (1979), that 5HT can stimulate an acute increase in plasma aldosterone levels (over an hour)

independent of other factors including PRA and K^+ . Whether 5HT increases plasma aldosterone through a central and/or peripheral mechanism at the level of the adrenal is still not clarified. As indicated in other studies, 5HT exerts important neural influences on the secretion of hypothalamic and pituitary factors such as CRF, β -endorphin, β -LPH, ACTH; these aldosterone stimulating factors and others such as α -MSH, β -MSH, γ -MSH and ASF, also exist within the pituitary and hypothalamic area, which is rich in serotonergic innervation (Petraglia et al., 1984) and may be subject to serotonergic control. The pituitary also contains 5HT containing cells and a decarboxylase capable of converting 5HTP to 5HT, so central 5HT may be involved in a paracrine role in controlling pituitary-adrenal axis steroidogenesis. Shenker et al., 1985 showed that oral administration of 5HTP into humans increased plasma aldosterone levels but peripheral inhibition of its conversion to 5HT did not alter the increase in plasma aldosterone, which was independent of PRA, K^+ or ACTH levels. They suggested a central serotonergic involvement in aldosterone secretion which may also be an important aetiological factor in the hypersecretion of aldosterone seen in primary aldosteronism. Gross et al., 1981 had previously shown that cyproheptadine (a 5HT antagonist) given to hyperaldosteronism patients, reduced plasma aldosterone levels. In idiopathic hyperaldosteronism, the sensitivity to AII and responsiveness is increased, and the extrapolation that this is due to higher 5HT concentration within the brain and/or periphery, could

account in part, for the some of the in vitro findings in this study when 5HTP increases plasma, and possibly adrenal levels of 5HT, and potentiated the response to AII and 5HT at the higher doses. Indeed, a neural component has been postulated in controlling the sensitivity and pulsatility of corticosterone secretion at the adrenal with ACTH (Ottenweller et al., 1978,1982), so that a central neural serotonergic element which controls the 'tonic' aldosterone output, is a possible factor in aldosterone biosynthesis. Alternatively, diurnal disruption of ACTH known to occur with PCPA administration, with an increase in the a.m. low ACTH and prevention of the p.m. rise in ACTH secretion, may affect steroid biosynthesis and basal and stimulated levels. In this regard an important in vivo and in vitro study undertaken by Vernikos-Danellis's group in 1973 indicated that after a single dose or two daily doses of PCPA in rats, the plasma corticosterone levels decreased and there was a parallel drop in in vitro corticosterone output in basal incubates as well as a reduced response to exogenous ACTH both in vivo and in vitro. Serotonin concentration within the brain was reduced to 20% the control values and there was an associated disruption in the circadian ACTH rhythm of secretion. Thus, although the changes in 5HT within the brain appeared to be an important controlling factor in adrenal responsiveness, the effects were not specific for the zona glomerulosa and aldosterone biosynthesis, but rather an ACTH- (and /or related peptides of the precursor hormone POMC), related effect on adrenal status. The effects of a single dose of PCPA on diurnal

changes lasts for 48 hours although 5HT levels remain low, so there is a clear dissociation between the two effects. However in these studies, rats were sacrificed 24 hours after the last PCPA injection, so that diurnal changes may in part have been responsible for the observations.

An effect of 5HTP administration in the opposite direction on diurnal rhythms was also observed by Popova et al., 1972, when they administered 5HTP to normal and hypophysectomised rats, in an attempt to localise the serotonergic effects to either peripheral or central mediated mechanisms. Hypophysectomy abolished the increase in plasma corticosterone seen in control rats after 5HTP administration, suggesting that the effect of 5HTP and 5HT are centrally mediated, at least in the control of adrenal corticosterone biosynthesis; by another means of increasing local concentrations of 5HT, Fuller et al., 1980, administered a specific 5HT releasing drug from neurones, parachloroamphetamine (pCA) into conscious rats, and during the time study plasma corticosterone levels increased within an hour of the injection, returning to basal values within 4 hours. Here again, it appears that 5HT generally has a stimulatory effect on corticosterone but this does not rule out the possibility that 5HT can specifically stimulate aldosterone in the zona glomerulosa by a central and/or peripheral mechanism, which was not specifically looked for in the studies on diurnal ACTH regulation and its relation to corticosterone biosynthesis. A possible peripheral component of 5HT's effects was suggested by Van de Kar et al., 1985 in which pCA, a drug that

releases 5HT stores, was administered to rats, and 2 hours later the rats were sacrificed and plasma corticosterone levels measured. By selective manipulations that included severing afferent connections to the hypothalamus from dorsal and medial raphe; mediobasal cuts in the hypothalamus which severed connection between the hypothalamus and pituitary; and hypophysectomy which removed pituitary influences, 5HTP induced changes in plasma corticosterone secretion even after hypophysectomy and thus pituitary influence. 5HTP administration acutely increased plasma corticosterone slightly but significantly. Unfortunately in this study, plasma aldosterone levels were not measured, the inference made is that 5HT and/or its precursor, may have a small but significant peripheral (and possibly direct) effect at the level of the adrenal cortex in altering steroid secretion. Similarly, in the in vivo and in vitro results above, manipulation of 5HT levels appear to alter adrenal responsiveness, and the lack of effect on plasma aldosterone levels in vivo could be a result of a more acute effect not seen over this time-scale. In the early sections of this chapter, measurements of 5HT in the adrenal were given which by any standards is high in tissues with high established serotonergic content e.g. brain approximately 1.5-2 μ g/g wet weight in some regions, such as the dorsal and raphe nuclei (Osborne 1982), so the possibility exists that 5HT is a locally stored amine within the adrenal; further, the metabolism studies, (Section 9.3, Chapter 9), indicate some storage of exogenous 5HT within the inner cortex and medulla. In addition therefore, 5HT may be taken up as an exogenous

source, from the circulating platelets and stored in the medulla /cortex in the same way that has already been postulated for dopamine in defining a paracrine role influencing 'tonic', and AII stimulated steroidogenesis. In other tissues, such as the hypothalamus, Jones (1977) showed that 5HT in a dose range of 10^{-9}M - 10^{-6}M , could stimulate in a dose dependent way, the release of CRF and serotonergic innervation and control of CRF release is well established. That 5HT also stimulates steroidogenesis within this dose range in the adrenal cortex, could be extended to a serotonergic neural involvement here, but to date there is no evidence to suggest this. Serotonin concentrations may also regulate and/ or modulate other possible local regulators such as dopamine, which could play a more direct role in the effects seen here. Thus, in PCPA treated rats, decrease in 5HT levels within the adrenal may reduce a possible fine control between dopamine and 5HT in controlling the release of somatostatin, which is known to inhibit plasma aldosterone secretion through well characterised receptors in the rat adrenal. Aguilera (1981) had previously suggested a dopaminergic inhibition of aldosterone mediated through somatostatin release which could then interact with well characterised somatostatin receptors in reducing aldosterone biosynthesis. The possibility exists of extending this fine control of somatostatin regulation through 5HT-dopamine ratios, in that high 5HT:DA ratios would inhibit its release and vice versa for high DA:5HT levels. Another serotonergic involvement in the control of other regulatory

factors of aldosterone includes a modulatory role on α -MSH release, particularly in low sodium status, or in the pathophysiological state of idiopathic hyperaldosteronism. In the latter instance, the aetiology includes enlargement of the pars intermedia, which contains several pituitary peptide fragments including α -MSH; in low sodium too, Vinson et al., 1980 indicated that α -MSH levels are increased in low salt status. One could speculate that in both instances, an increase in serotonergic stimulation to the hypothalamic and pituitary regions could induce these changes in α -MSH level which are known to stimulate aldosterone secretion in the low salt state (Whitehouse et al., 1982). That sodium per se may alter the sensitivity of the adrenal to aldosterone stimulating factors including All, and in some reports, to ACTH (Oelkers 1974., Kinson et al., 1968) is recognised, but that 5HT concentrations may be altered, or that sensitisation changed as a result of changes in 5HT levels is discussed more fully in Chapter 10.

Other factors were described in the introductory chapters as modulating the steroid output both in vivo and in vitro. PCPA may have depleted to some extent catecholamine levels within the medullary tissue and affected steroid biosynthesis; similarly in the case of 5HTP administration, newly synthesised 5HT can be achieved by displacement of endogenous catecholamines within medullary granules and/or catecholaminergic sympathetic neurones, so the possibility exists that 5HTP indirectly increases the local concentration of catecholamines within the adrenal, which potentiates steroid

biosynthesis in a non-discriminate way. The salient feature of these studies lies in the observations that altering 5HT levels with these drugs, may affect plasma corticosterone, aldosterone and PRA acutely, but that the in vitro responsiveness of the zona glomerulosa cells is altered chronically, even when other parameters are not significantly changed within the two groups on the same day of sampling. Thus the events appear to be dissociable from the effects of other circulating factors including ACTH. The consequences of altered responsiveness and possibly sensitivity in vivo, could be investigated further, by infusion studies of AII, ACTH and potassium and comparing the dose response of plasma aldosterone in PCPA/5HTP treated and control rats. Further studies of the effects of changing endogenous 5HT levels on the dose response to AII infusions in altered salt diets may point to a possible modulatory role of 5HT and/ or dopamine in altering adrenal sensitivity. A peripheral involvement of 5HT levels in modulating plasma aldosterone levels could also be dissociated from central effects by using hypophysectomised rats. That 5HT is intimately involved at some level in the 'tonic' control of aldosterone biosynthesis, and moreover, the likelihood of it's direct local stimulatory effect, is credible given the reproducible dose response curves with incubation of cells with exogenous 5HT within the physiological range 10^{-9} M.

Chapter 9

THE METABOLISM OF SEROTONIN IN THE ADRENAL

Although histochemical techniques are useful in indicating 5HT storage, metabolic studies that follow the pathway of 5HT or its precursors 5-hydroxytryptophan (5HTP) or tryptophan, provide a dynamic picture of storage and turnover. One of the first studies undertaken to follow 5HT storage in the whole animal was done by Bertler et al., 1960 in which rabbits were injected i.v. with 5HTP and the amount of 5HT formed in the adrenal medulla was measured by differential centrifugation. Serotonin was localised in the medulla in concentrations of 1.4µg /adrenal pair and furthermore appeared within cytoplasmic granules rather than within the cytoplasm itself. It was suggested that in this species at least, the adrenals contain enzymes capable of converting 5HTP to 5HT. Further to these precursor studies Gershon et al., 1966, injected radioactive precursors [³H]-5HTP or [¹⁴C]-5HTP into mice, demonstrated by autoradiography 'active' sites of 5HT turnover and this allowed for the measurement of tissue radioactivity, and chromatography of products formed. 5-hydroxytryptophan differs from 5HT in that it penetrates the brain, liver, the gastrointestinal tract and any other organ that contains the enzyme aromatic amino acid decarboxylase. Moreover, the ability of cells to accumulate 5HT from 5HTP appears to be linked to the endogenous levels of 5HT. The relationship is reciprocal in that cells with large amounts of endogenous 5HT accumulate less amine, compared to those with lower amounts of endogenous 5HT. Gershon

(1966) found high concentrations of radioactive 5HTP in the adrenal which was rich in decarboxylase activity but poor in 5HT content. Although there is evidence for storage of 5HT in the medulla (Holzwarth et al., 1983., Verhofstadt et al., 1983., Kuriyama et al., 1984). In early studies, Gershon (1966) showed that there did not appear to be a rapid turnover of 5HT in the whole adrenal gland, with administered radioactive 5HT remaining in the gland for 72 hours. Furthermore, 90% of the [3 H]-5HT remained in the adrenal compared to 58% and 41% in the spleen and lung respectively. There were no detectable metabolites occurring in adrenal tissue compared to 13% and 14% metabolites in the spleen and lung respectively and in these studies, it appeared that the adrenal gland was capable of converting the maximal amount of precursor 5HTP to 5HT. Despite this evidence for precursor requirement for 5HT synthesis, it is not known how much circulating 5HTP is readily available for uptake.

More recent in vitro studies were carried out by Trost et al., 1976 in which decapsulated and capsular tissue from bovine adrenals were incubated at 37⁰C in the presence of [3 H]-5HT and the accumulation of [3 H]-5HT and/or its metabolites measured by TLC and liquid scintillation counting. Trost's work indicated that [3 H]-5HT was taken up, both by the capsular and decapsular tissue, and compared to other tissue fragments, the highest proportion of radioactivity was found in the capsular portion of the adrenal gland and the aorta with a 'ceiling' effect of accumulation in the aorta and the decapsular portions after 1 hour, suggestive of what Gershon had called 'the

endogenous 5HT binding site pools.' In contrast, 5HT accumulation continued to rise in the capsular portions during the second hour of incubation. In decapsulated tissue, [³H]-5HT was taken up independent of increasing concentration of the amine, until concentrations of 10⁻³M-10⁻²M were reached, whereas in the capsular portion tissue radioactivity fell with increasing [³H]-5HT; the capsular tissue converted 3-4 times more 5HT to 5HIAA per unit weight than the decapsular portions of tissue incubates. The fractional retention of radioactivity varied with incubation time and the initial 5HT concentration. From these studies it appeared that the capsular tissue did not store the amine. More recent studies by Holzwarth (1983) failed to identify any 5HT containing neurones, which also argued against 5HT storage in capsular and outer cortical tissue. However, 5HT has been measured in rat adrenal medullary tissue by HPLC (Verhofstadt et al., 1983., Kuriyama et al., 1984) and localised by immunohistochemistry techniques (Verhofstadt 1983.,).

Vaccari et al., 1977 measured the levels of tryptophan hydroxylase, the rate limiting enzyme in 5HT biosynthesis and L-aromatic amino acid decarboxylase in several brain regions and the adrenal glands of male and female rats at different stages of development. In these studies it was found that in 60 day old adult rats tryptophan hydroxylase levels in the adrenal gland were 0.43nmol/hr/g protein in females compared to 0.34nmol/hr/g protein in males. This compares to some regions of rat brains of similar age e.g. mesodiencephalon where the levels are 0.36nmol/hr/g protein in

females and 0.16nmol/hr/g protein in males. Furthermore L-aromatic amino acid decarboxylase in the adrenal occurs with values of 18.3±2.6 μ mol/hr/g protein in females compared to 29.2 μ mol/hr/g protein in males. In the pons brainstem region where the activity of this enzyme is considered to be high, the values for female rats average 6.9 μ mol/hr/g protein and 7.8 μ mol/hr/g protein in male rats. Monoamine oxidase (MAO) is also found in the adrenal at levels of approximately 14.6 μ mol/hr/g protein in both females and males. MAO activity can be found in both the adrenal medulla and the cortex and in studies carried out by Youdim in 1974 (cited in Youdim 1976) MAO activity was more concentrated in the cortical tissues of rat, pig and dog compared to medullary tissue. The presence of L-aromatic amino acid decarboxylase and monoamine oxidase (MAO) in the adrenal does not prove that 5HT acts there as a neurotransmitter, however the presence of the rate limiting enzyme, tryptophan hydroxylase, does suggest that de novo synthesis of 5HT in some region of the adrenal, more likely the medulla, does occur in some species including the rat.

The purpose of the present studies was to continue the work of Trost in determining the metabolic fate of added radioactive [3 H]-5HT in isolated cells from the inner decapsular and outer capsular regions of the rat adrenal. The advantage of using isolated cells in this work was the removal of connective tissue and associated mast cells that in the rat and mouse are capable of uptake of 5HT. Also, a direct comparison of the fate of 5HT in glomerulosa cells compared to

fasciculata/reticularis cells could be made although contaminating medullary tissue was also likely to be present in the latter case. Further, studies examined the steroidogenic potency of indoleamines and 5HT metabolites as possible stimulatory factors for steroidogenesis in the zona glomerulosa.

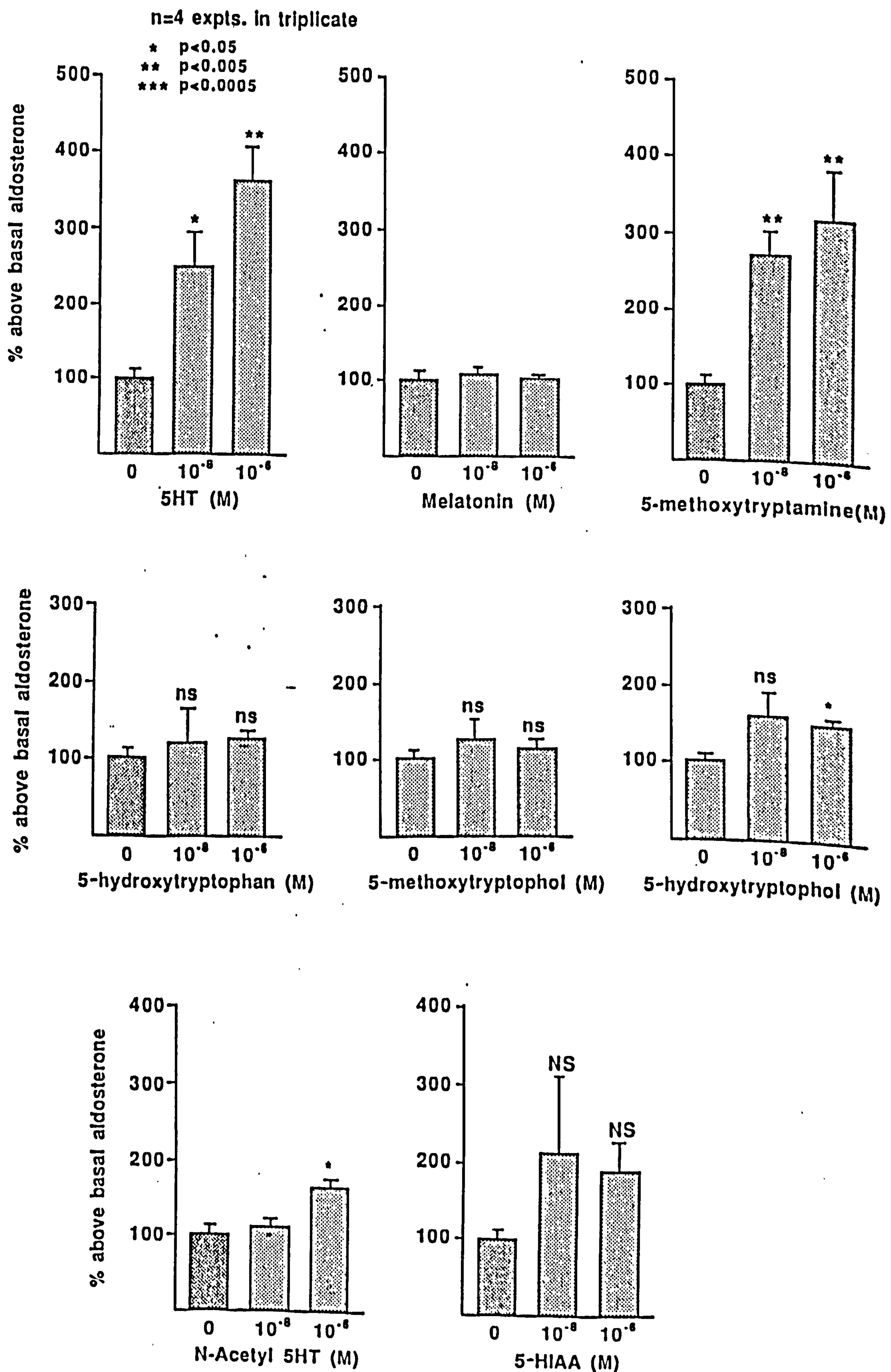
9.2 STEROID RESPONSE OF GLOMERULOSA CELLS TO SOME INDOLEAMINES

Previous work by Al-Dujaili et al., 1982 using rat zona glomerulosa cells in a perfusion system showed that 5HT, 5-methoxytryptamine (5MT) and to a lesser extent 5-methoxytryptophol and melatonin were able to stimulate aldosterone secretion. 5-Methoxytryptamine appeared to be even more potent than 5HT in this respect. Muller in 1970 was able to show a stimulatory effect of 5HT, 5MT and L-5-hydroxytryptophan in static incubates of adrenal slices, at doses of 10^{-6} M for all but the latter compound which had a stimulatory effect only at 10^{-5} M.

9.2.1 The Effects of Indoleamines on Aldosterone Secretion

Further to this work these amines as well as some metabolites of 5HT i.e. 5-hydroxyindoleacetic acid (5HIAA), N-Acetyl-5HT, 5-hydroxytryptophol and other related indoleamines including 5-methoxytryptophol and melatonin were all incubated at two doses, 10^{-8} M and 10^{-6} M with isolated rat zona glomerulosa cells in the method described for cell incubations in Chapter 6. The rationale behind this study was: (1) to determine how specific 5HT stimulated steroidogenesis is in relation to other structure-related indoleamines and to define more clearly the structure-activity

Fig 9.1
% stimulation above basal of aldosterone with several indoleamines
in the zona glomerulosa cell



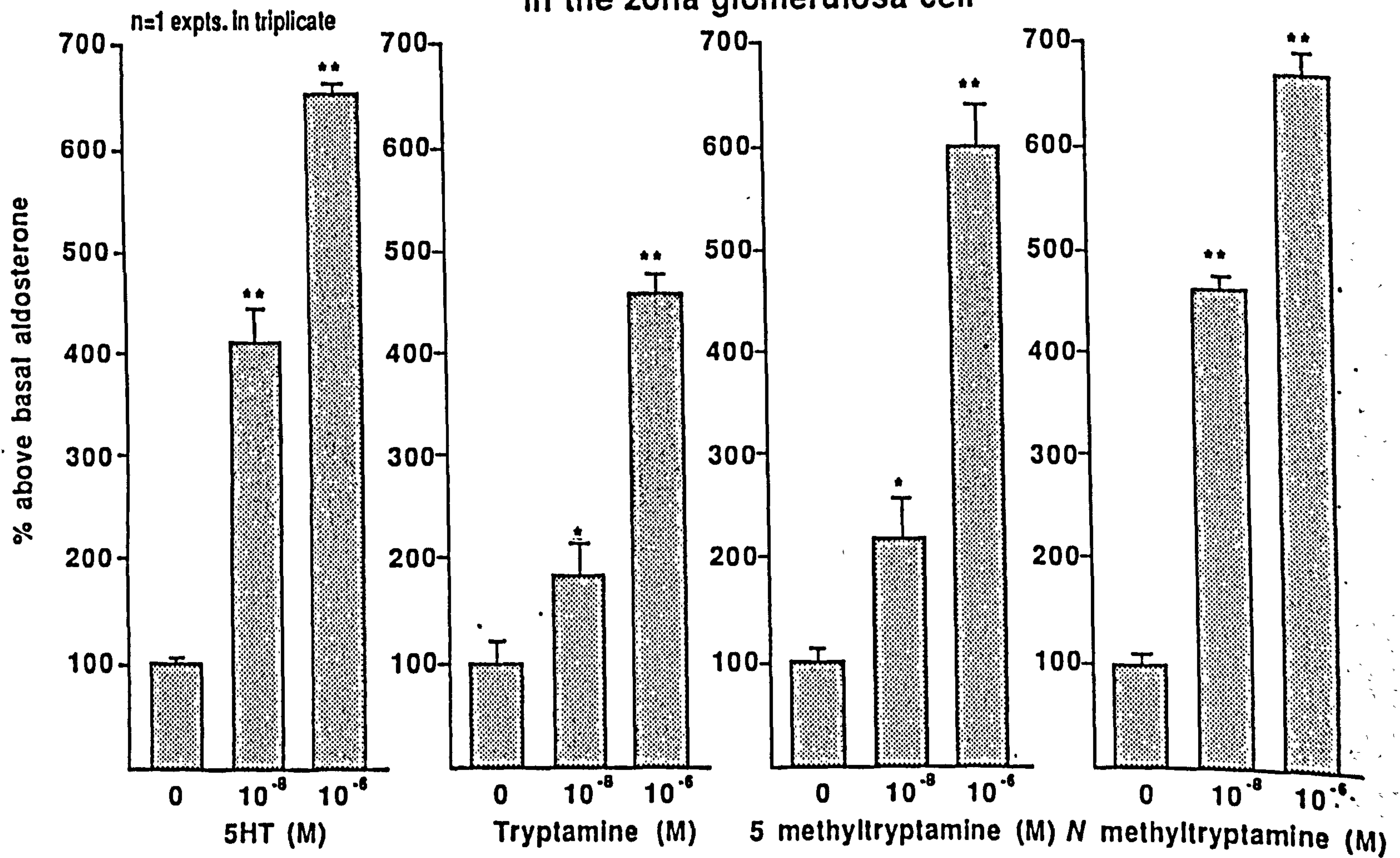
requirements for this stimulation in the zona glomerulosa cells; (2) to see if the metabolite(s) or intermediates produced during monoamine oxidase activity on 5HT rather than the molecule itself is responsible for the steroidogenic response .

Between two to four experiments were done using female adult Wistar rats and 10^5 cells per incubate were prepared. Stock solutions of the indoleamines ($10^{-3}M$) were prepared in saline or absolute alcohol and the final alcohol concentration never exceeded 0.1% in the cell incubates. Serial dilutions ten times greater than the final concentration in the incubation medium were made in Medium 199 (0.2% BSA). The steroid output varied between experiments (this is possibly due to different sensitivity of receptors during enzyme digestion and preparatory treatment on different days). All results therefore are expressed as percentage values of aldosterone relative to the control in 'n' number of experiments. The results were analysed statistically using Student's unpaired t-test.

9.2.2 RESULTS

Figure 9.1 shows the percentage above basal aldosterone output in 4 experiments for two doses of indoleamines compared to the reference amine, 5HT at $10^{-8}M$ and $10^{-6}M$ in the zona glomerulosa preparation. 5HT significantly enhanced the aldosterone output to 250% ($p < 0.05$) at $10^{-8}M$ 5HT and 350% ($p < 0.005$) at $10^{-6}M$ 5HT. 5-Methoxytryptamine approached the potency of 5HT in elevating aldosterone at 275% at $10^{-8}M$ and $> 300\%$ at $10^{-6}M$. The indoleamines melatonin, 5HTP, 5-

**Fig 9.2 % Stimulation above basal of aldosterone with several indoleamines
in the zona glomerulosa cell**



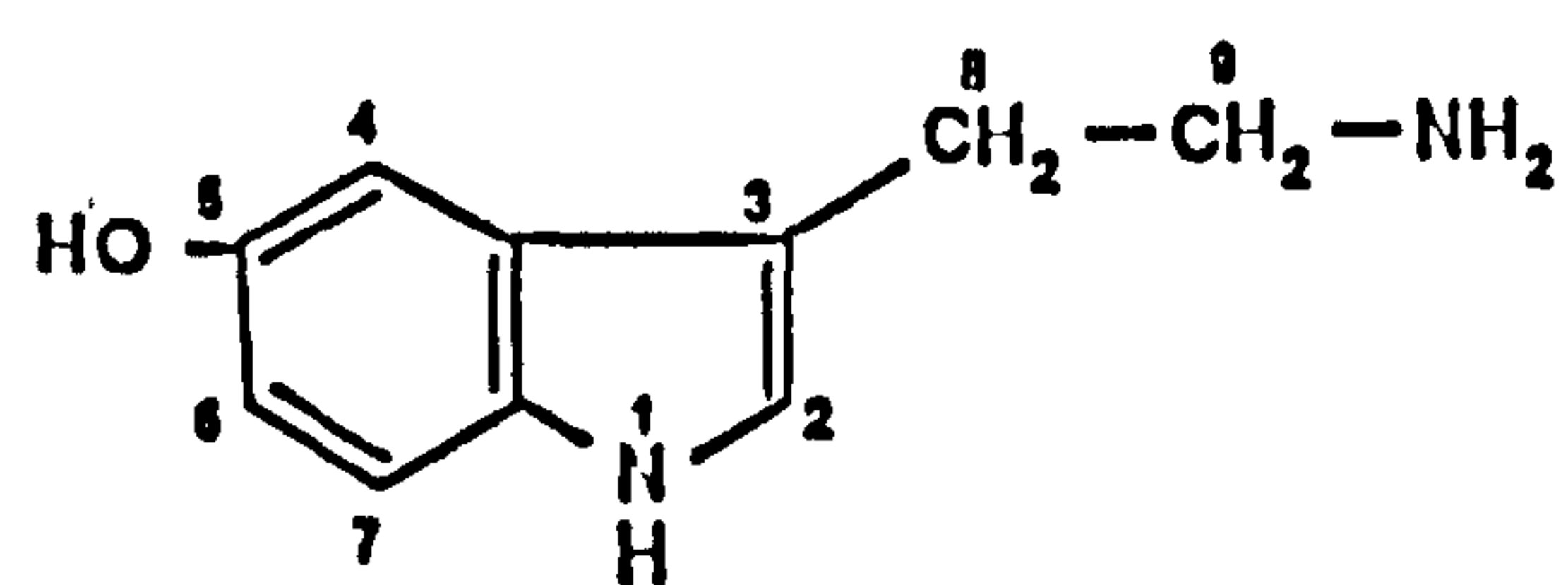
methoxytryptophol and 5HIAA did not significantly stimulate aldosterone at either dose. N-Acetyl-5HT stimulated to approximately 150% aldosterone output at 10^{-6} M ($p < 0.05$) and 5-hydroxytryptophol stimulated aldosterone output to 175% at 10^{-8} M, but this was not significant possibly because of the larger standard error bars in this experiment although the stimulation became significant at 10^{-6} M at 150% above basal ($p < 0.05$).

Figure 9.2 shows the results of 1 experiment. Tryptamine, 5-methyltryptamine and N-methyltryptamine all significantly elevated aldosterone at both doses (10^{-8} M- 10^{-6} M) ($p < 0.05$). At the lower dose of 10^{-8} M, N-methyltryptamine approached a similar potency to 5HT. Tryptamine and 5-methyltryptamine were approximately equipotent and half as potent as 5HT or N-methyltryptamine.

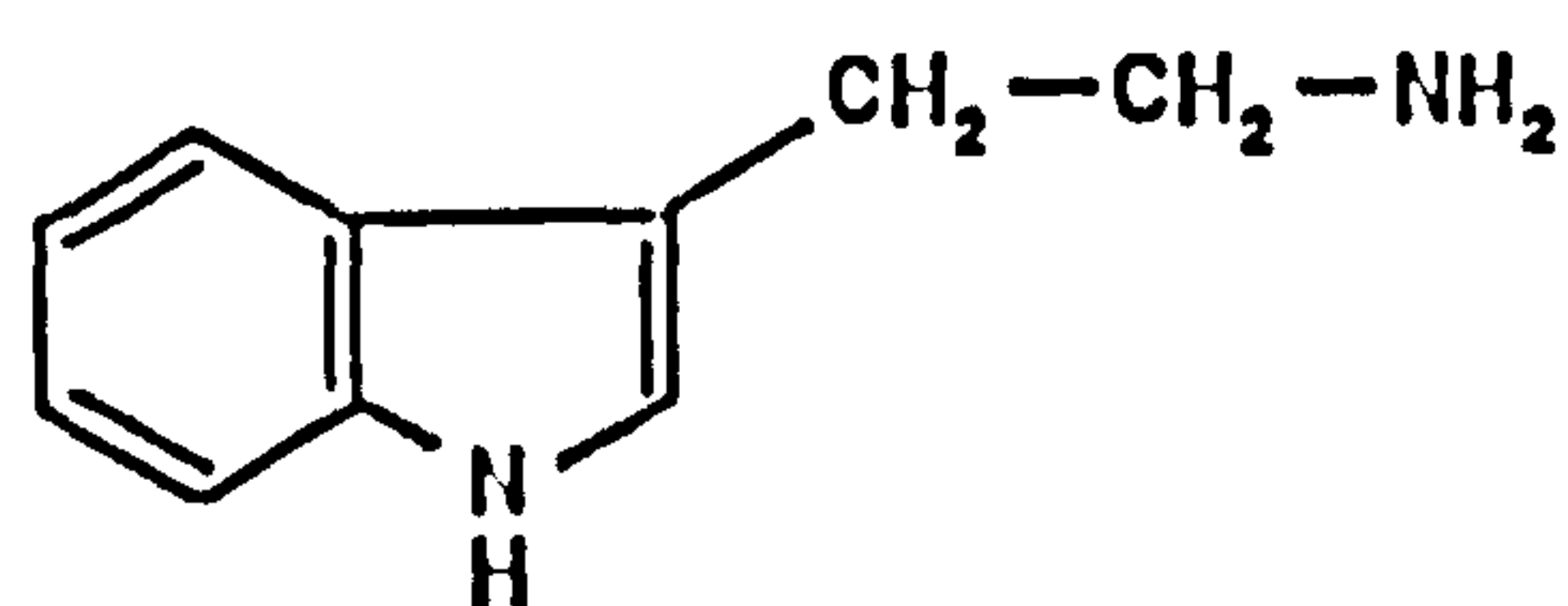
9.2.3 DISCUSSION

From the graphs it can clearly be seen that the indoleamines with a free amino group at position C9 or with a methyl group attached to the nitrogen (N) atom (Fig.9.3) are the most potent stimuli of aldosterone biosynthesis. Thus, increasing the basic character at this position and therefore the electron cloud negativity appears to be of primary importance in aldosterone secretion. Substitution as in 5MT and an unaltered amino portion, with a retaining of some of the basic character at this site, i.e. inclusion of an oxygen atom, does not alter the potency of indoleamine stimulated aldosterone production, and substitution with

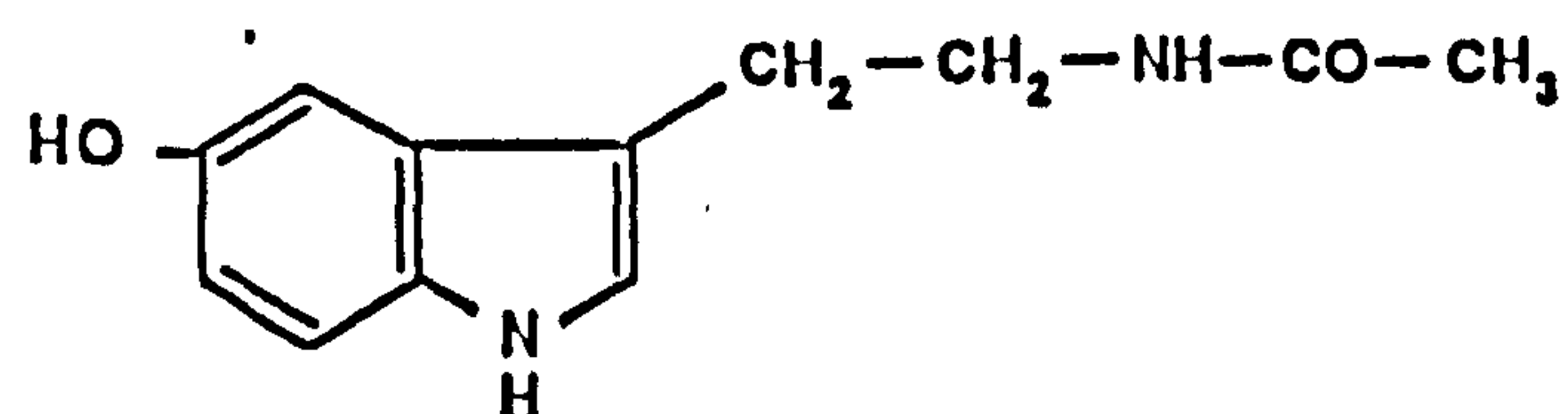
Fig 9.3 Structures of several Indoleamines



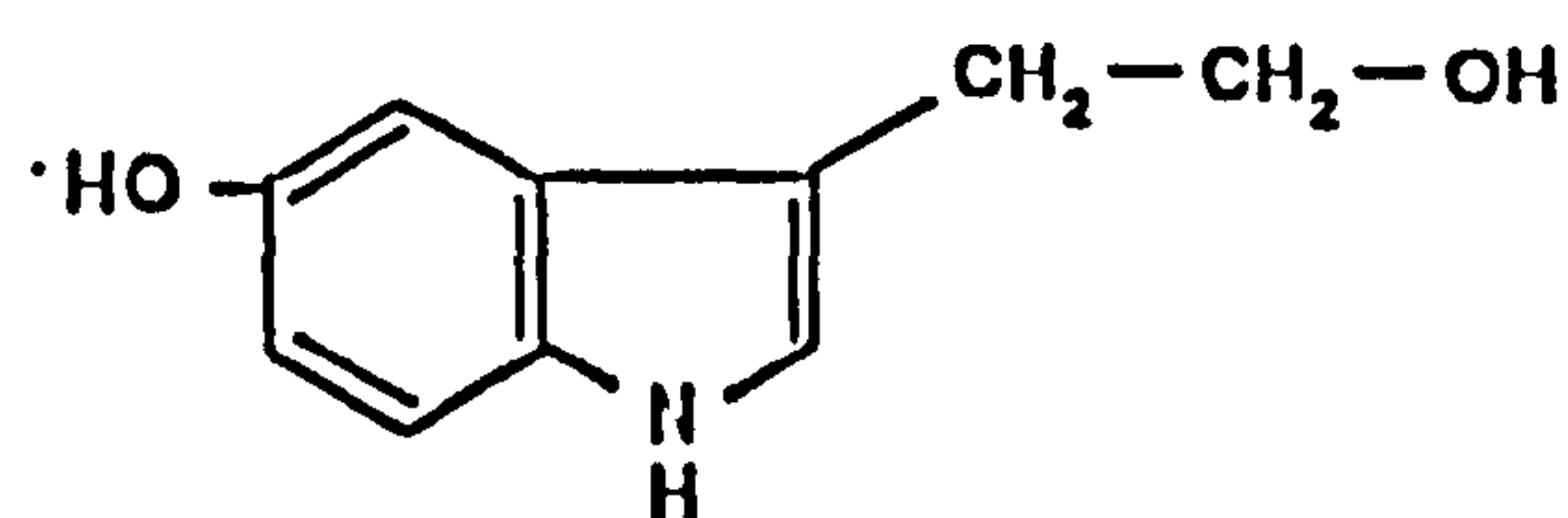
5HT



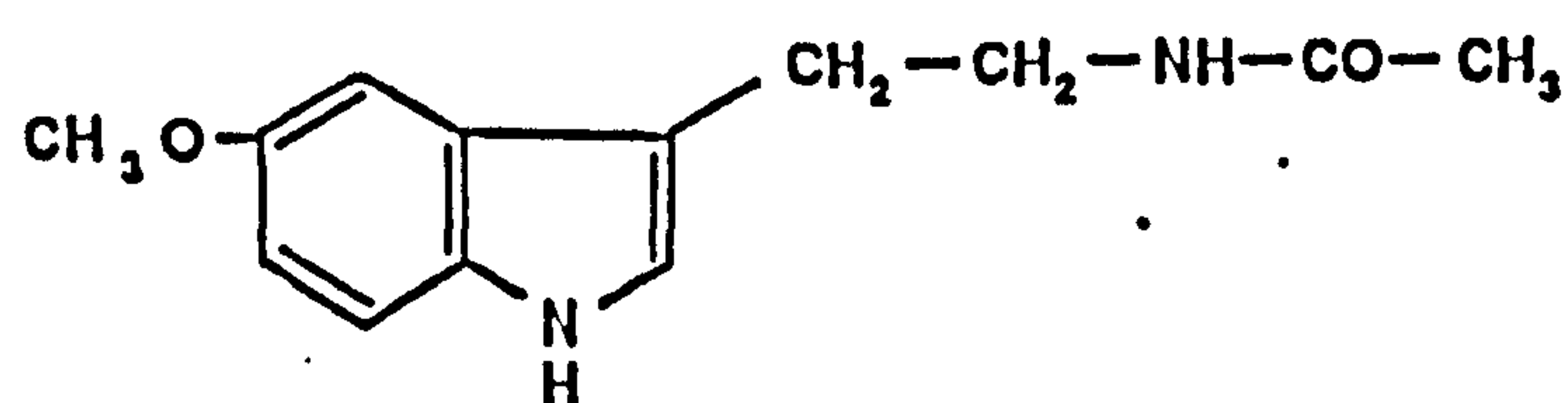
Tryptamine



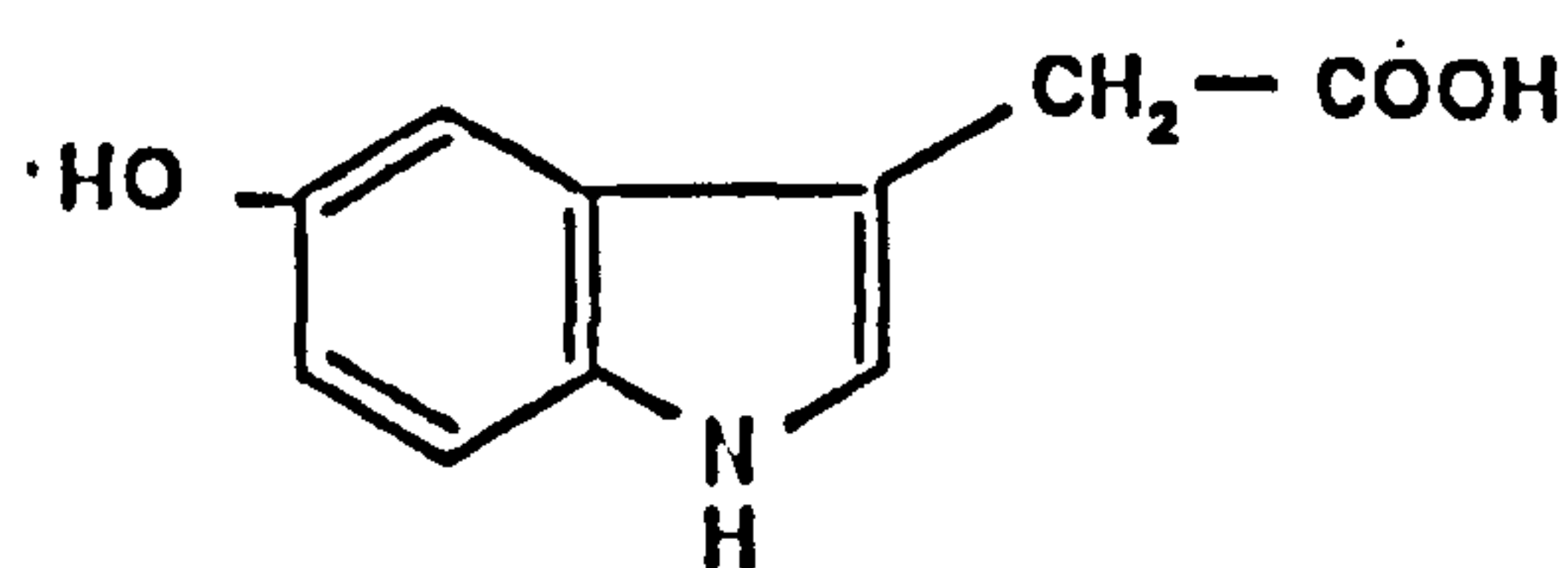
N-Acetyl 5HT



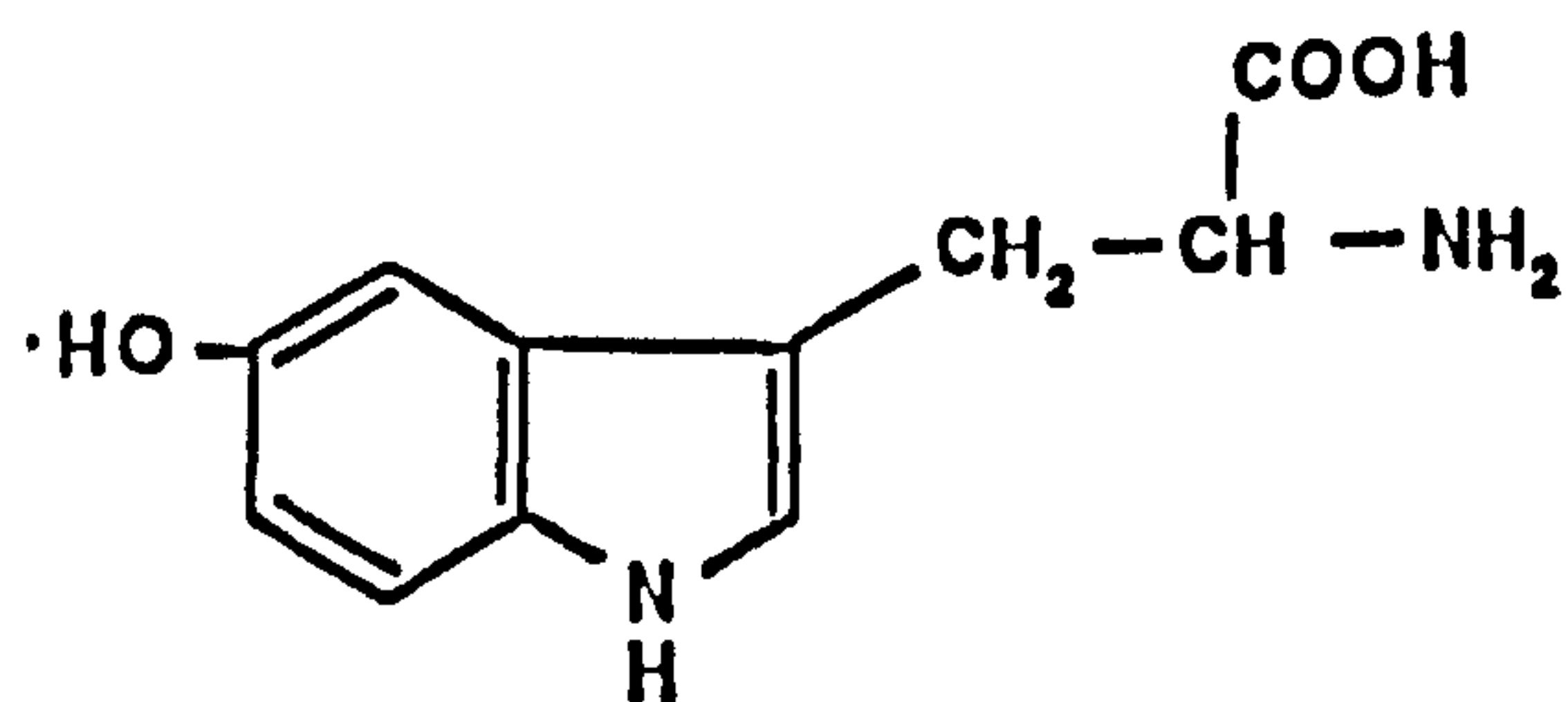
5 Hydroxytryptophol



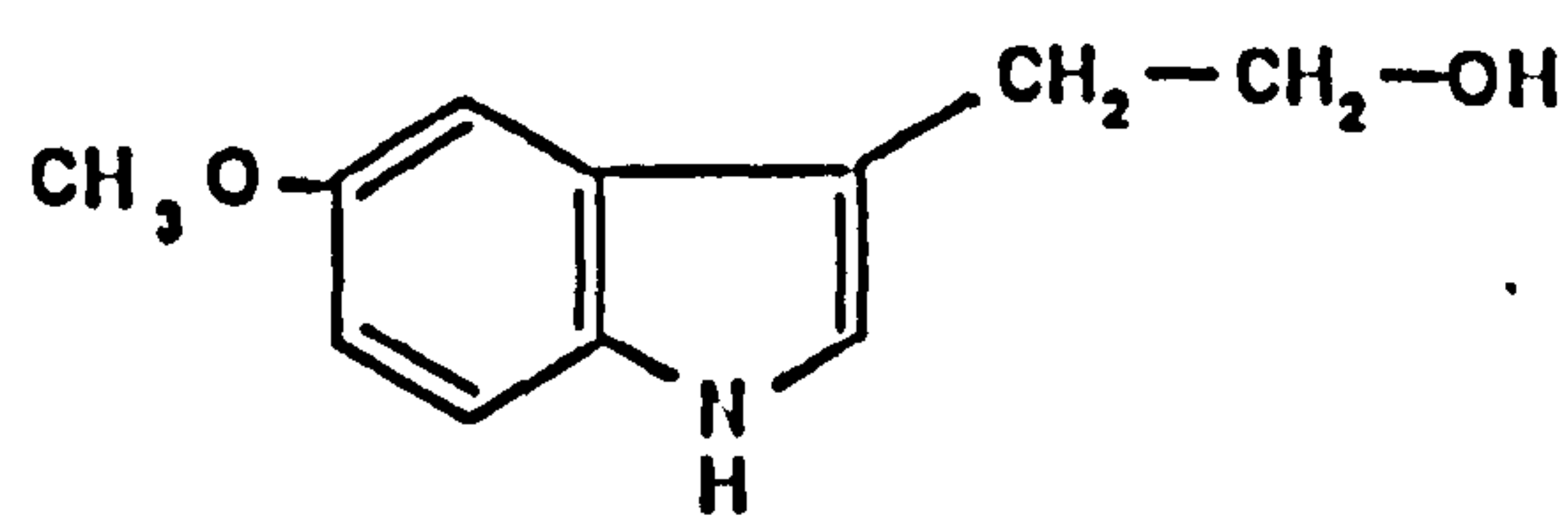
Melatonin



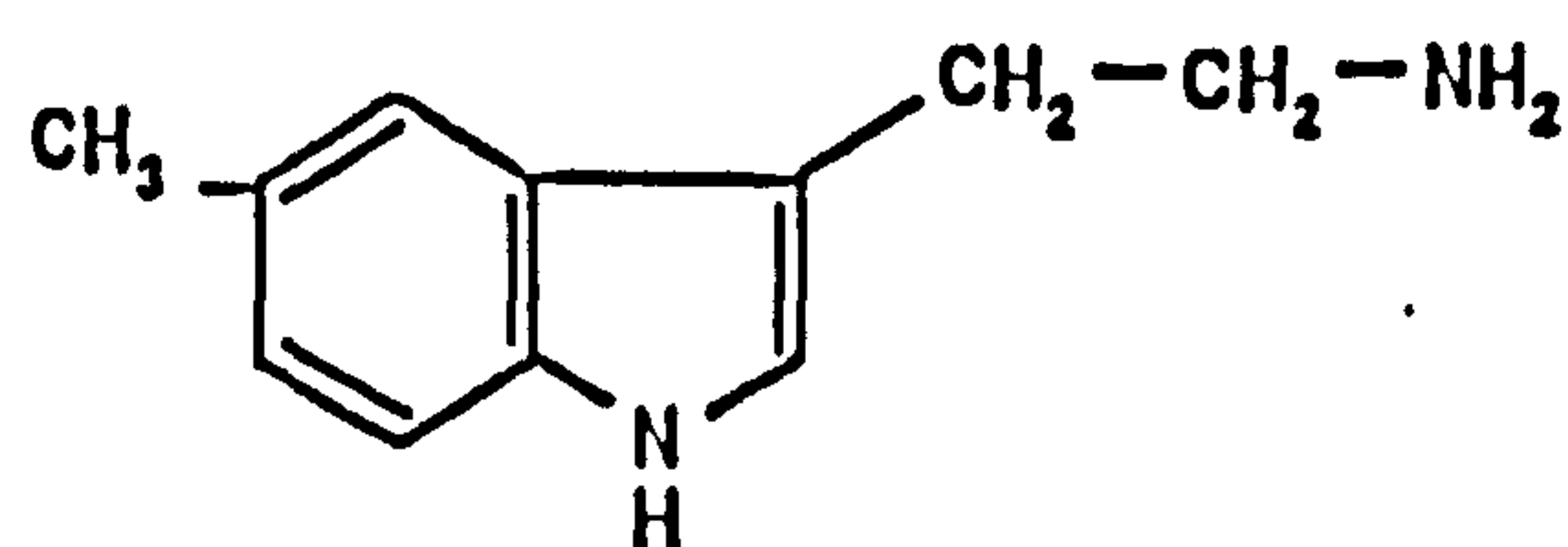
5 - Hydroxyindole acetic acid



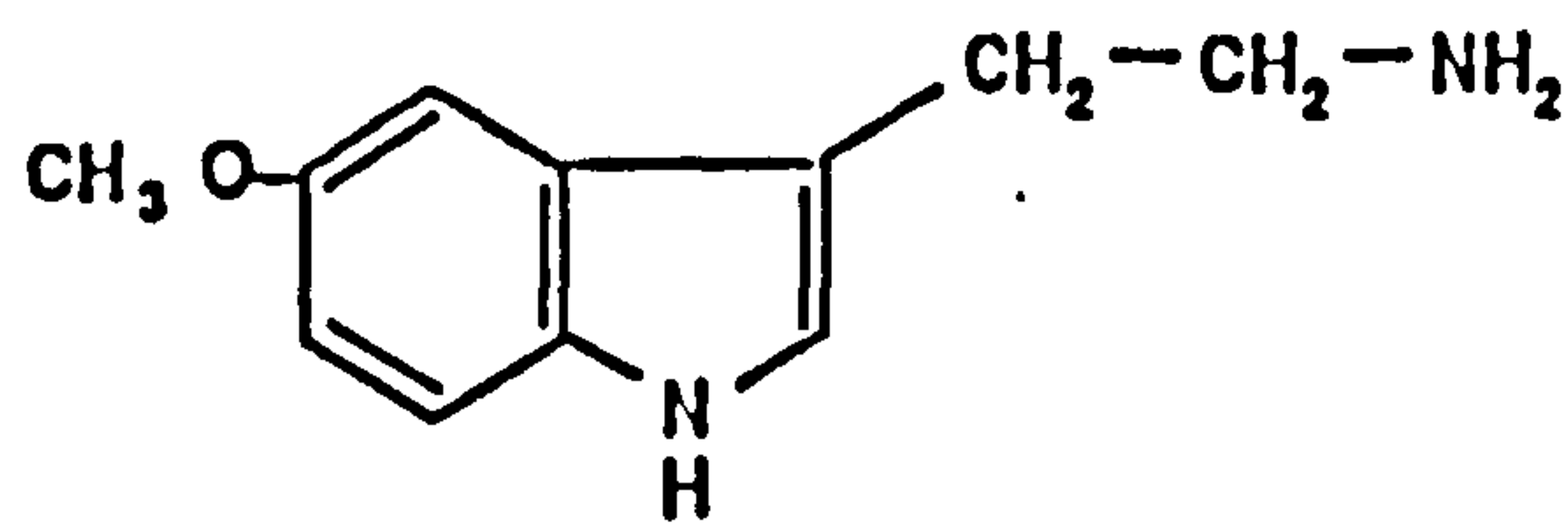
5 - Hydroxytryptophan



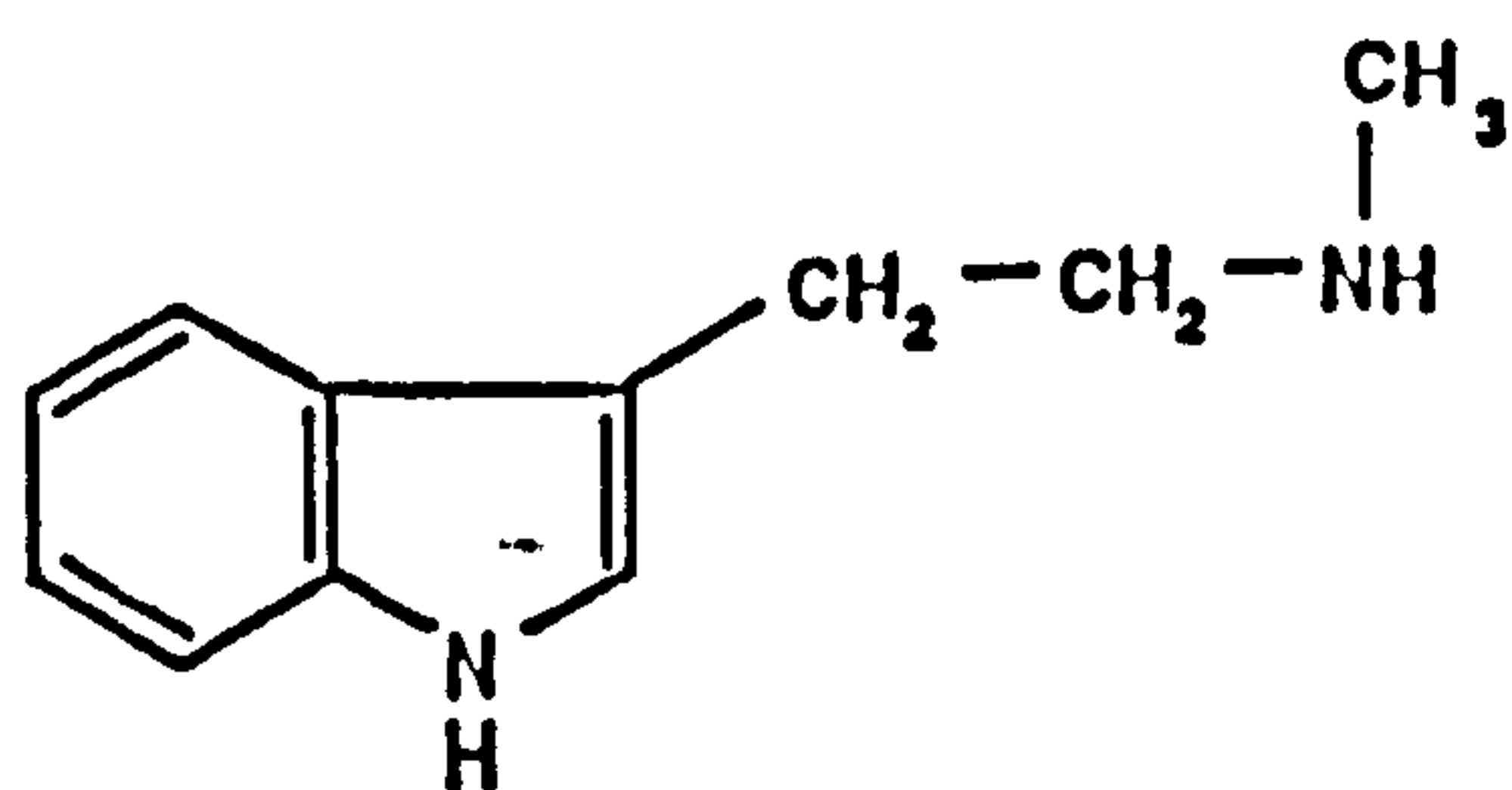
5 - Methoxytryptophol



5 - Methyltryptamine



5 - Methoxytryptamine



N-Methyltryptamine

a non-basic group such as 5-methyl 5HT, or indeed complete removal of the group at position C5, e.g. tryptamine, slightly reduces the potency of the stimulation. Thus, the indole moiety appears to be of primary importance in stimulation of aldosterone secretion.

Modification of this region e.g. N-Acetyl-5HT or 5-hydroxytryptophol allows for slight stimulation possibly because of the presence of the other important 5OH region, but the common feature of reduced basic character, reduces potency. In this respect, modification of this region too, (e.g. melatonin and 5-methoxytryptophol), has no stimulatory effect, unlike Al-Dujaili's findings, which employed a column perfusion system.

The requirement of intact ethylamine structures in these studies would tend to suggest that if a 5HT type receptor exists within the zona glomerulosa, it requires this group for steroidogenesis as opposed to binding per se. Previously, it had been shown in studies by Niles et al., 1983, that the hydroxyl group possessed by 5HT and N-Acetyl-5HT were essential for high affinity binding in the rat brain and since N-Acetyl-5HT interacted with the 5HT receptor, it was suggested that there was a close interaction on the same 5HT receptor; however it was shown by Cohen et al., 1985, that tryptamine could interact with 5HT receptors in the rat fundic region, so the conformational requirement for stimulating through 5HT receptor sites does not appear to be too rigid. In a series of binding studies for 5HT and its structural requirements in brain tissue, Bennett and Snyder 1976 showed that the key requirement for 5HT₁ high affinity

binding was the 5-hydroxy substituted indole moiety and this was supported by the studies of Allgren et al., 1985 using solubilised bovine cortical brain membranes. The latter group also observed that membranes specifically required potent interactions between 5-hydroxy and a free amino terminal group for high affinity binding and this was lost in solubilising the membranes. Allgren postulated that the 5HT₁ site may exist as a subunit complex containing separate amine and indole attachment sites.

From the observations it could be suggested that a 5HT binding site with type 1 character may exist, and at higher concentrations of 5HT adenylate cyclase is increased as suggested for type 1 receptors; further investigation into the effects the stimulatory indoleamines have on cAMP levels and the use of more specific 5HT₁ receptor antagonists are required to complete the picture of 5HT binding sites in the adrenal and the subclassification of a type 1 receptor.

9.3

METABOLISM STUDIES

9.3.1 Cell Preparation

Isolated zona glomerulosa and decapsulated tissue for the preparation of zona fasciculata cells was prepared as explained in chapter 6. Tritiated 5HT was obtained from Amersham International plc., and had a specific activity of 77.3mCi/mg. The molecular weight of the creatinine salt was 405 and the radioactive concentration was 1.0mCi/ml.

The purity of the product was assessed by injecting a solution of 10µl in 10ml of 0.15M perchloric acid onto the HPLC column and

measuring the counts in 1ml/minute fractions in a beta counter in relation to the retention time for a 5HT standard solution. It was found to be approximately 98% pure.

A stock solution of the label was made by addition of neat label, 50 μ l to 10ml of incubation buffer Medium 199 (0.2% BSA) containing 10^{-5} M cold 5HT.

An aliquot of the stock solution, 100 μ l was added to 5ml of cocktail Scintran to determine the counts and thus the original concentration of radioactive 5HT added to the incubates.

10^6 cpm were present in 100 μ l aliquot (original stock)

This volume was added to 0.9ml incubates thus = 10^6 cpm/incubate

The beta counter used had a counting efficiency of 32% for ^3H thus - 3.125×10^6 DPM, which corresponds to a concentration of 4.5 pmol/ml added to each tube.

This amount of radioactivity was dissolved in a stock solution of cold 10^{-5} M 5HT in 0.2%BSA.

9.3.2 Incubation of Cells

In the control experiments isolated cells or medium alone, were pre-incubated for 15 minutes in M199 (0.2% BSA), whilst pargyline, added at a final concentration of 10^{-5} M was added to additional aliquots of medium (+ cells) for 15 minutes preincubation. After this period 100 μ l additions of mixed label + cold 5HT was added to all tubes and incubated for a further 30 minutes. All tubes were then briefly placed on ice to stop the reactions and incubates centrifuged at 4°C for 15minutes at 15,000g.

9.3.3 Cell Pellets and Medium Preparation for HPLC

A 400 μ l aliquot of the medium supernatant was pipetted into clean conical polypropylene tubes containing 50 μ l 15% perchloric acid and 50 μ l internal standard for indoleamines (N- ω -methyl-5HT) as previously described in Chapter 6.

After sample preparation a 25 μ l aliquot was injected onto the HPLC column and 1ml/min fractions collected in plastic scintillation tubes containing 5mls of cocktail Scintran for a total run time of 20 minutes. Previous standards for 5HIAA, 5HT, N-methyl-5HT, N-Acetyl-5HT, 5-methoxytryptamine, 5,6-dihydroxytryptamine and 5,7-dihydroxytryptamine were injected onto the column to determine their retention times.

All cpm fractions corresponding to the retention times of 5HT, 5HIAA and other possible metabolites, were integrated and subtracted from background counts. In the final calculation all cpm values in the medium were corrected up for total deproteinising volume of 400 μ l in 500 μ l i.e.5/4 and multiplied up by a factor of 1000 μ l/25 (injection volume) = x 40

The remaining medium was discarded and the cell pellets washed twice in 500 μ l of ice cold saline. Finally the cell pellets were deproteinised in 50 μ l of 1.5% perchloric acid containing 1 μ M internal standard and left to stand for 15 minutes at 4⁰C before sonication for 10 seconds per tube at speed setting 6 on ice to completely disrupt the cells and thus release any stored labelled 5HT.

The cell homogenate was centrifuged for 15 minutes in a refrigerated

Experiment 1

Table 9.1

Glomerulosa Cells

Sample Number	(A)	(B)	(C)	(D)	(E)
	³ H5HT dpm x10 ⁶ ml ⁻¹ medium	³ H5HIAA dpm x10 ⁶ ml ⁻¹ medium	³ H5HT dpm x 10 ⁵ cell pellet ⁻¹	³ H5HIAA dpm x 10 ⁵ cell pellet ⁻¹	T count x 10 ⁶ dpm
3	2.62	0.28	3,360	1,540	2.90
4	2.54	0.195	2,520	1,800	2.75

Table 9.2

Fasciculata Cells

Sample Number	(A)	(B)	(C)	(D)	(E)
	³ H5HT dpm x10 ⁶ ml ⁻¹ medium	³ H5HIAA dpm x10 ⁶ ml ⁻¹ medium	³ H5HT dpm x 10 ⁵ cell pellet ⁻¹	³ H5HIAA dpm x10 ⁵ cell pellet ⁻¹	T count x 10 ⁶ dpm
3	2.30	0.29	47,950	5,020	2.65
4	2.52	0.32	45,630	8,400	2.91

Samples 3 & 4 = cells + ³H5HT + 10⁻⁶ M5HT cold.

All dpm values in 3 & 4 are the mean of original counts - dpm values background counts.

microfuge, and a 25 μ l sample injected onto the HPLC column.

Fractions were collected as for the medium, but the final cpms were corrected up to the total volume of the digest compared to the injected volume i.e.

$$50/25\mu\text{l} = \times 2/100,000 \text{ cells}$$

All values therefore in the tables are corrected for dilution factors and converted to DPM after preliminary determination of the counting efficiency for the beta counter.

9.3.4 Direct Radioimmunoassay of the Medium

Aliquots of control and stimulated cell medium \pm 10^{-5} M pargyline, were assayed for aldosterone secretion, as described in Chapter 6.

9.4 RESULTS

An example of the calculations are given for the first experiment. Tables 9.1 and 9.2 show the dpm values derived from correcting for dilution factors and recovery. Samples 3 and 4 in Table 9.1 show the dpm values for 5HT and 5HIAA in zona glomerulosa cells and cell medium without pargyline. Similarly, Table 9.2 shows the dpm values for zona fasciculata and inner cortical and medullary cells and medium. Tables 9.3 and 9.4, samples 5 and 6 show the dpm values for 5HT conversion to 5HIAA in medium alone. Thus these values and the subsequent calculation of percentage conversion of 5HT by air oxidation processes alone can be derived (see Table for calculation) and the percentage value subtracted from those values derived from glomerulosa cells and inner cortical/medullary cells.

Experiment 1

Table 9.3

Recovery of ^3H Counts in Medium Alone

Sample Number	$^3\text{H5HT}$ dpm $\times 10^6 \text{ ml}^{-1}$ medium	$^3\text{H5HIAA}$ dpm $\times 10^6 \text{ ml}^{-1}$ medium	$^3\text{H5HT}$ dpm $\times 10^5$ cell pellet	$^3\text{H5HIAA}$ $\times 10^5$ cell pellet	T counts $\times 10^6$ dpm
5	2.48	0.13	-	-	2.61
6	2.59	0.09	-	-	2.68

Table 9.4

Sample Number	% total $^3\text{H5HT}$ used
5	4.98
6	3.35
Mean \pm SD	4.16 \pm 0.8

Samples 5 & 6 $^3\text{H5HT} + 10^{-6} \text{ M5HT (cold)} + \text{medium}$.

Experiment 1

Glomerulosa Cells

Table 9.5

Sample Number	% total ³ H5HT used	% converted to ³ H5HIAA	% ³ H5HT in cell pellet (x 10 ⁵ cells)	% total ³ H5HT in cell pellet (x 10 ⁵ cells)
3	5.32	95.80	1.20	0.12
4	3.46	91.00	1.20	0.09
	4.39 ± 0.93	93.4 ± 2.4	1.20	0.105 ± 0.015

Fasciculata Cells

Table 9.6

Sample Number	% total ³ H5HT used	% converted to ³ H5HIAA	% ³ H5HT in cell pellet (x 10 ⁵ cells)	% total ³ H5HT in cell pellet (x 10 ⁵ cells)
3	10.00	81.80	13.70	1.90
4	9.20	82.82	11.70	1.60
	9.6 ± 0.4	82.3 ± 0.5	12.7 ± 1.00	1.75 ± 0.15

$$\% \text{ total } ^3\text{H5HT used} = (E) - (A) / (E) \times 100$$

$$\% \text{ converted to } ^3\text{H5HIAA} = (B+D) / E-A \times 100$$

$$\%^3\text{H5HT in cell pellet} = C/E-A \times 100$$

$$\%^3\text{H5HT (total) in cell pellet} = C/E \times 100$$

Values in columns 1 & 2 are derived from subtracting % ³H5HT (mean) used in samples 5 & 6 (-) cells.

Table 9.5 shows the resultant percentage values from incubating zona glomerulosa cells at 37⁰C for 30 minutes. Of all the [³H]-5HT available to the cells, only 5.32% and 3.46% in samples 3 and 4 respectively, of the total, is used by the cells themselves. This approximates 4.4%, and approximately 93% of this is converted to the metabolite 5HIAA, specifically by the cells rather than the medium alone. Only approximately 1.2% of the total [³H]-5HT used, remains in the glomerulosa cells (capsular region) after 3 saline washes (see also Fig.9.4).

Table 9.6 shows that approximately twice as much of the total [³H]-5HT available is used by the inner adrenal i.e. 9.6% per same number of cells compared to the capsular region. Of this, 82.3% is converted to [³H]-5HIAA; approximately 13% of the total [³H]-5HT used, is retained in the cell pellet, considerably more than in the zona glomerulosa (Fig.9.4). Thus, overall more 5HT is used by the inner adrenal cortex/medulla compared to the capsular region, and less is metabolised in inner zones relative to the zona glomerulosa. Hence, more 5HT appears to be 'retained' in the inner zones.

The second metabolism experiment (Fig. 9.5) included the same protocol as the first but also included tubes incubated (= pargyline) in medium alone or in the presence of cells. Roughly the same amount of labelled 5HT was added to both zones of cells but the percentage values suggest that more 5HT is used by the inner cortex compared to the zona glomerulosa cells. Although there is not a significant difference in the corrected percentage conversion of 5HT

Fig 9.4

Metabolism of ^3H 5HT in isolated zona glomerulosa and zona fasciculata/reticularis cells in vitro at 37°C for 30 minutes

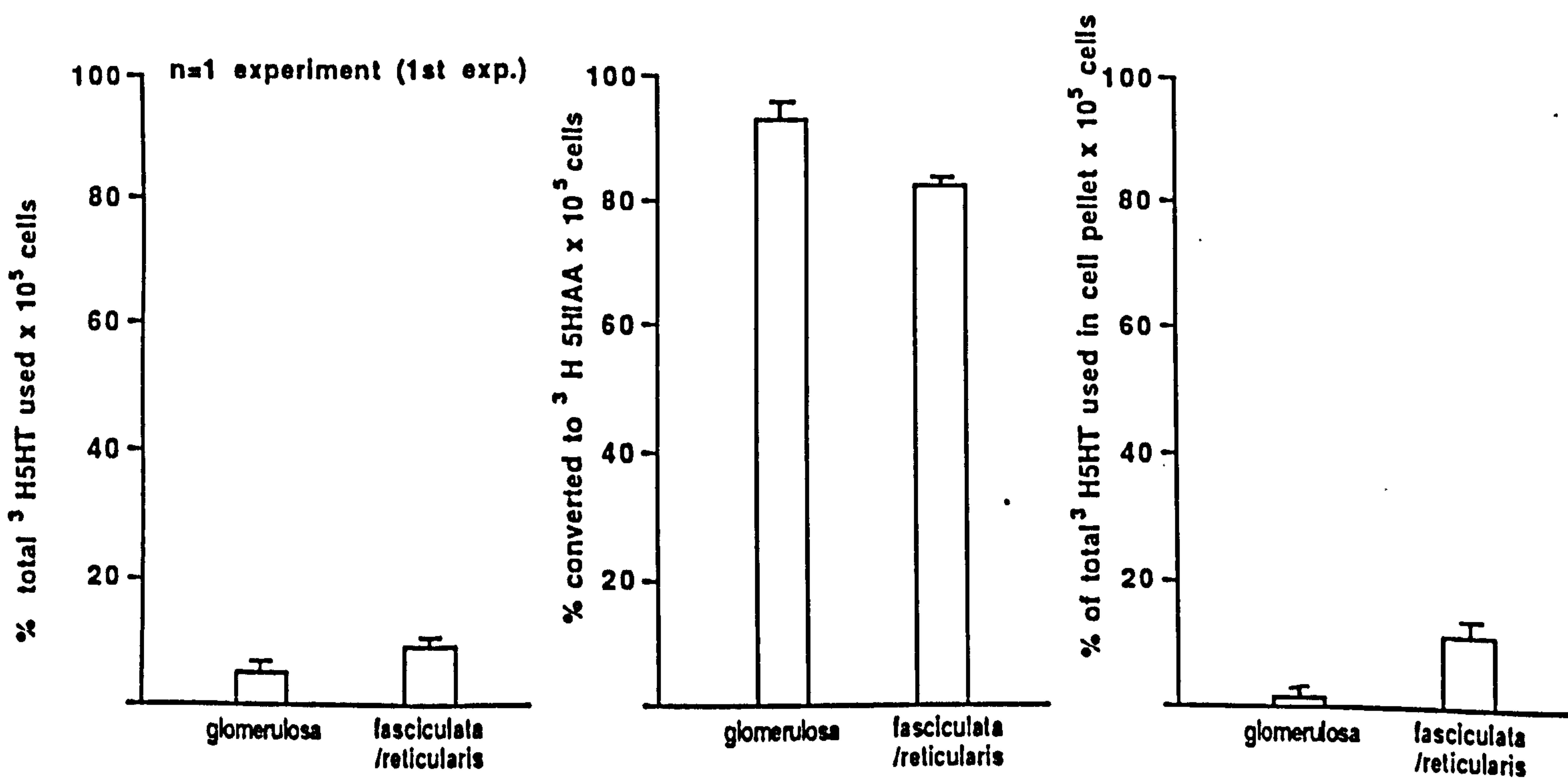
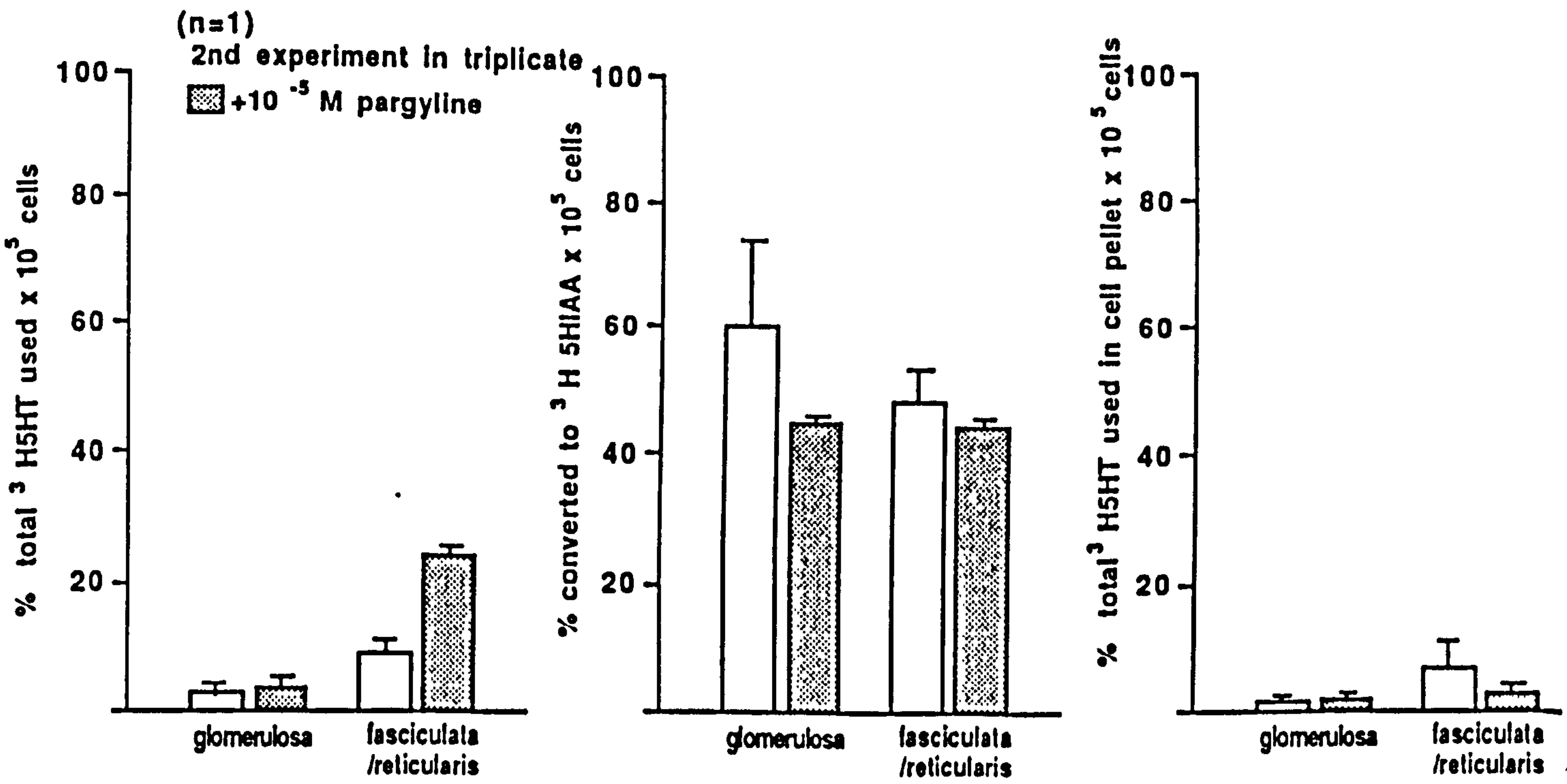


Fig 9.5

Metabolism of ^3H 5HT in isolated zona glomerulosa and zona fasciculata/reticularis cells in vitro at 37°C for 30 minutes



**Pooled Data for ³ H5HT Metabolism In vitro
in Isolated Cells at 37°C**

Table 9.7

Glomerulosa Cells

Sample	% total ³ H5HT used	% converted to ³ H5HIAA	% ³ H5HT cell pellet	% total ³ H5HT cell pellet
Exp 1				
3	5.32	95.80	1.20	0.12
4	3.46	91.00	1.20	0.09
Exp 2				
3	3.11	77.10	0.98	0.07
4	3.61	43.91	1.76	0.14
mean ± SD ± SEM	3.87 ± 0.85** ± 0.49	76.9 ± 20.3 ± 11.71	1.28 ± 0.28* ± 0.16	0.10 ± 0.03 ± 0.015

Table 9.8

Fasciculata Cells

Sample	% total ³ H5HT used	% converted to ³ H5HIAA	% ³ H5HT cell pellet	% total ³ H5HT cell pellet
Exp 1				
3	10.00	81.80	13.70	1.90
4	9.20	82.82	11.70	1.60
Exp 2				
3	7.68	53.61	5.40	0.64
4	10.41	41.81	8.70	1.29
mean ± SD ± SEM	9.32 ± 1.04** ± 0.60	65.01 ± 17.80 ± 10.30	9.87 ± 3.14* ± 1.87	1.36 ± 0.46 ± 0.27

* p<0.02

** p<0.001

Students paired t-test.

to 5HIAA in the zona glomerulosa ($60.51\% \pm 16.6$) compared to the in the inner zones ($47.7\% \pm 5.8$) the trend in the two samples suggests greater monoamine oxidase activity in the outer zones as seen in the first experiment (see Figs 9.4 and 9.5).

In percentage analysis the amount of [^3H]-5HT used by the zona glomerulosa cells is not significantly different in either experiment without pargyline, though less is converted to 5HIAA in the second experiment (60.5%) compared to the first (93%). However from the results measuring 5HT retained in the cell pellets after 3 washes, the amount retained in the zona glomerulosa is approximately the same (see discussion).

The pooled percentage data for the capsular and inner adrenal zones (Table 9.7 and 9.8 and Fig.9.6) shows these results graphically.

The overall percentage use of the total [^3H]-5HT available is approximately 2.5 times as great in the inner zones per 10^5 cells than in the zona glomerulosa cell preparation. The overall percentage conversion of the total [^3H]-5HT used is not significantly different in the two zones although there is a tendency for greater metabolism in the zona glomerulosa. Overall, significantly more 5HT is retained in the inner zones compared to the zona glomerulosa cells ($p < 0.02$).

In the second experiment (Fig. 9.5) a further set of tubes were incubated with/without pargyline (10^{-5}M). The total amount of 5HT used in the zona glomerulosa is not significantly changed by the presence of pargyline; the amount converted to 5HIAA with pargyline

Fig 9.6
Metabolism of ^3H 5HT in isolated zona glomerulosa
and zona fasciculata/reticularis cells in vitro at 37°C for 30 minutes

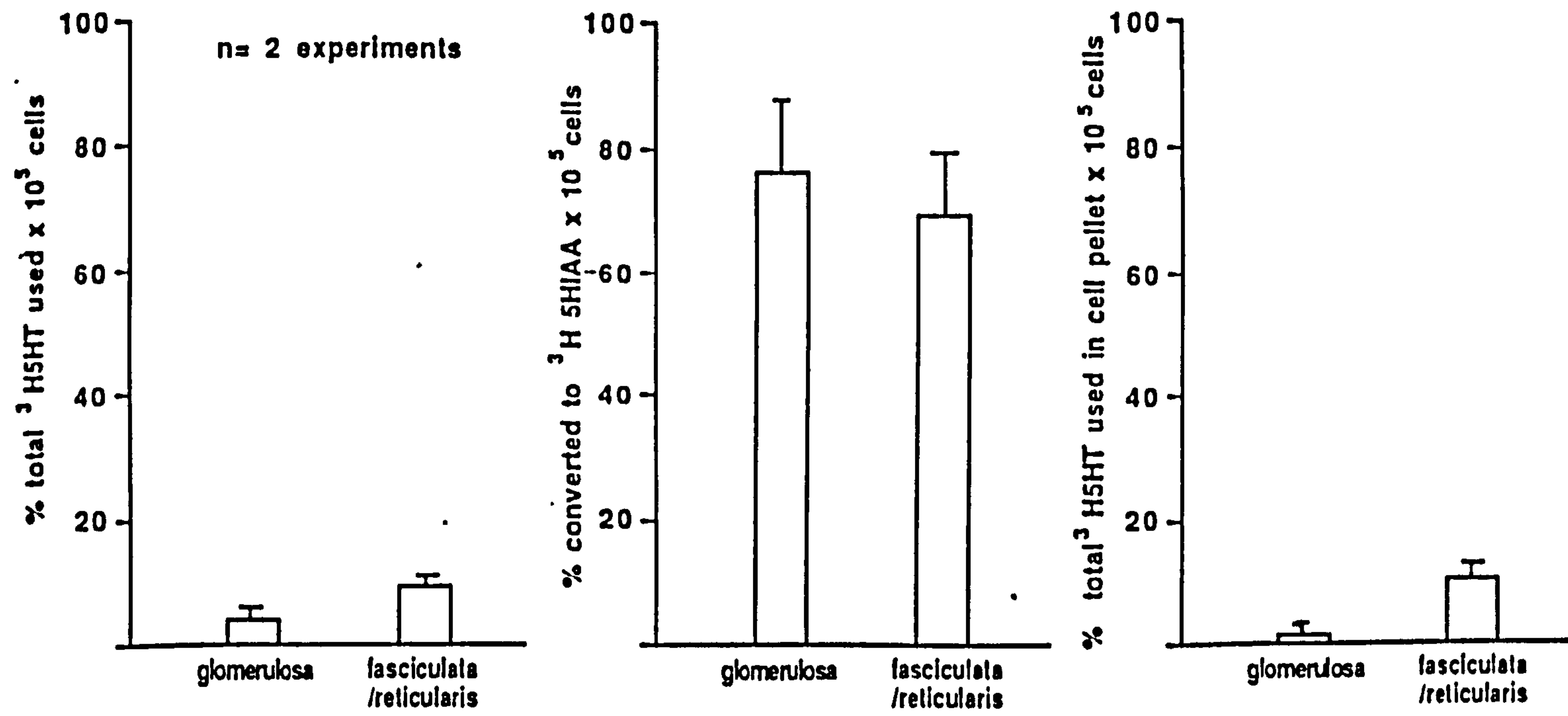


Fig 9.7a

Profile of ^3H counts in supernatant of glomerulosa cell incubates \pm Pargyline

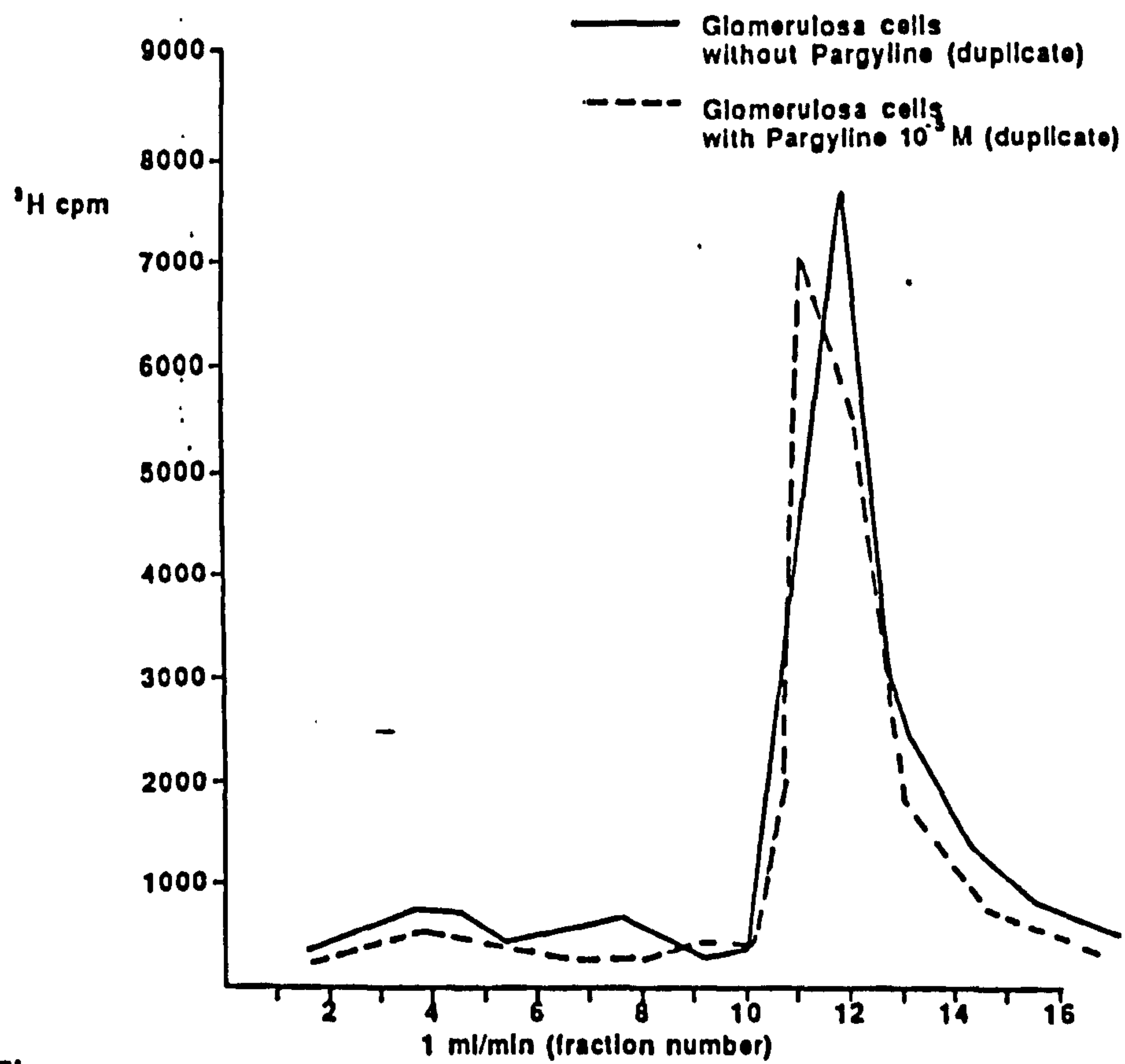
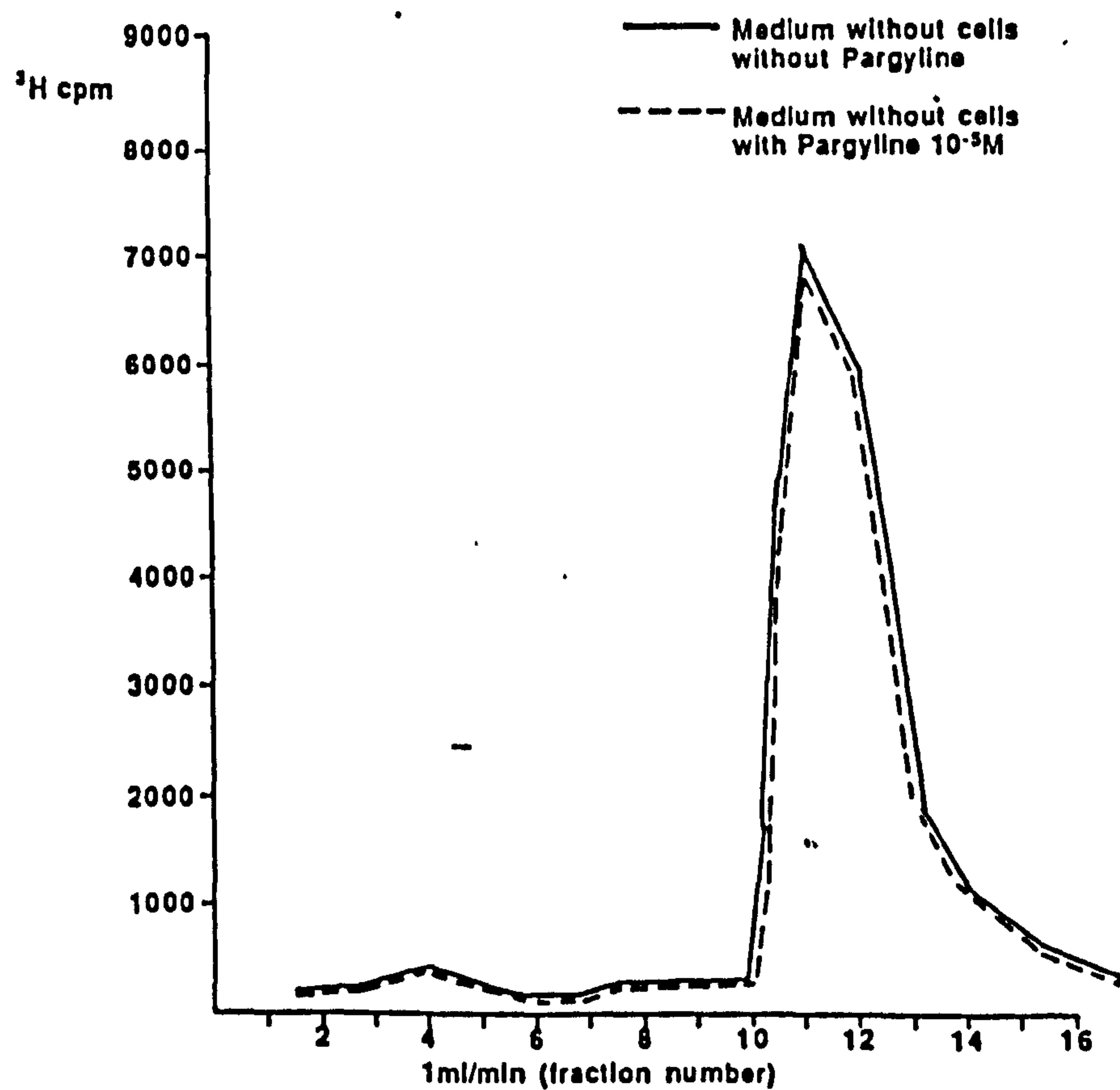


Fig 9.7b

Profile of ^3H counts in supernatant of incubates without cells \pm Pargyline



Mobile phase 1ml/min

R 5HT = 10 mins

R 5HIAA = 7.4 mins

R $N\omega$ -methytryptamine (Internal std.) = 14.15 mins

present is reduced from 60% to approximately 45% although statistically this was not significant. In the same experiment the raw data counts (cpm) for HPLC collected fractions in medium and cells + medium with pargyline present (Fig.9.7 a and b) shows that pargyline removes the 5HIAA peak (retention time (Rt) 6.5 mins - broken line) from the medium incubated with cells present. The amount of 5HT retained in the washed cell pellet is not significantly different in the presence of pargyline. Figure 9.9 (b) shows that pargyline slightly reduces the low level of counts at the 5HIAA peak (Rt 5.4 minutes - closed circle).

Inner zones incubated in the presence of pargyline use significantly more of the total [³H]-5HT available, i.e. 9.04% used (- pargyline) compared to 24% used (+ pargyline) see Figure 9.5. However, there is no significant difference in the amount converted to 5HIAA in the presence of pargyline. This percentage result is anomalous with the raw data cpm values obtained for aliquots of medium, where the 5HIAA peak is removed in the presence of pargyline (Fig.9.8 a and b). Considerably more 5HT appears to be retained in the cell pellet (- pargyline) i.e. 7% compared to only 2.15% (+ pargyline) - see Fig.9.5. The results in raw counts (Fig. 9.9 (a)) also show a slight elevation of the 5HT peak in the presence of pargyline, which is contradictory to the final recovery and corrected dpm values calculated. The final analysis does however show a trend towards inhibiting 5HIAA formation, in the inner adrenal zones treated with pargyline, in the same way as the capsular data demonstrates.

Fig 9.8a

Profile of ^3H counts in supernatant of fasciculata cell incubates \pm Pargyline

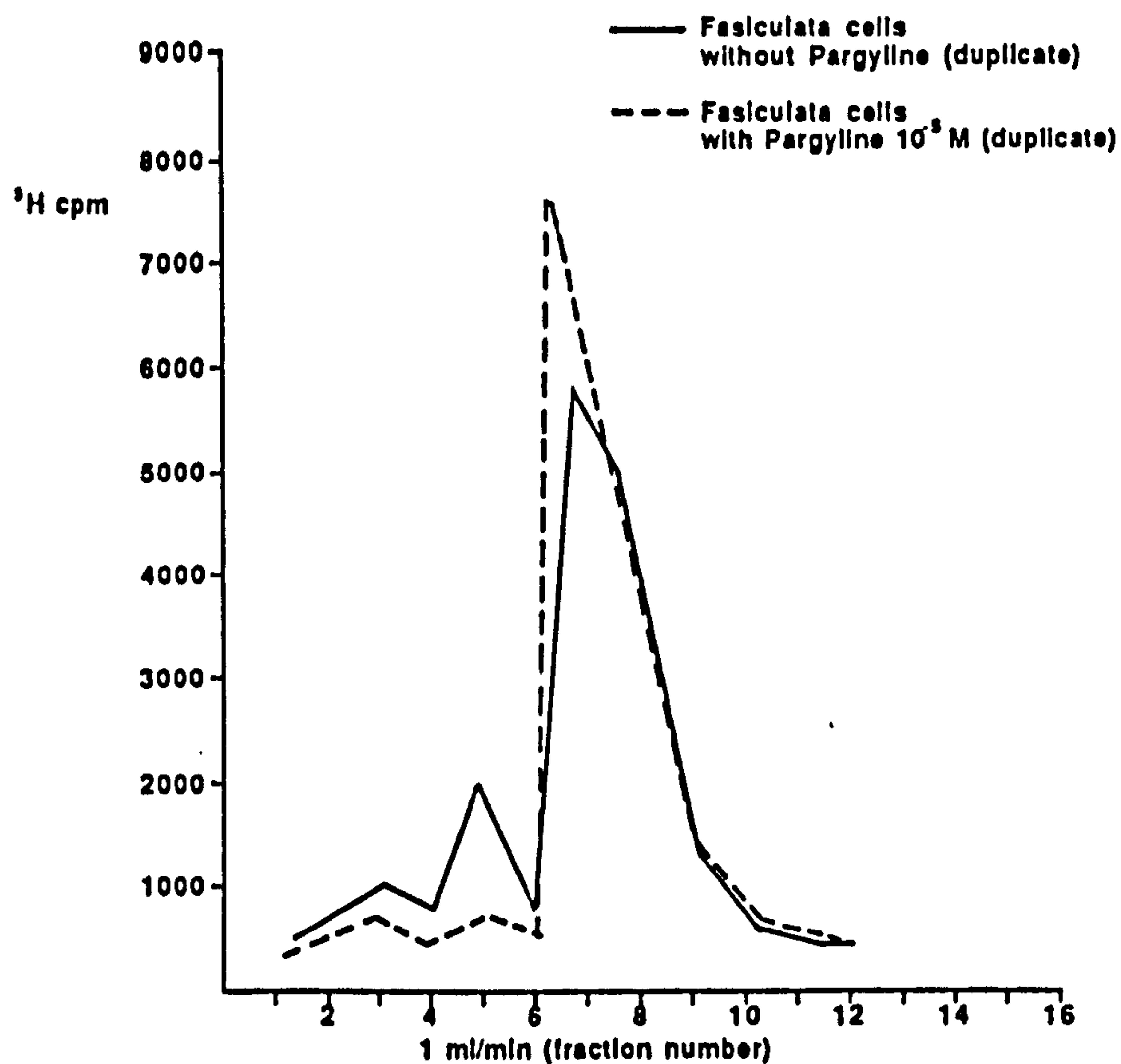
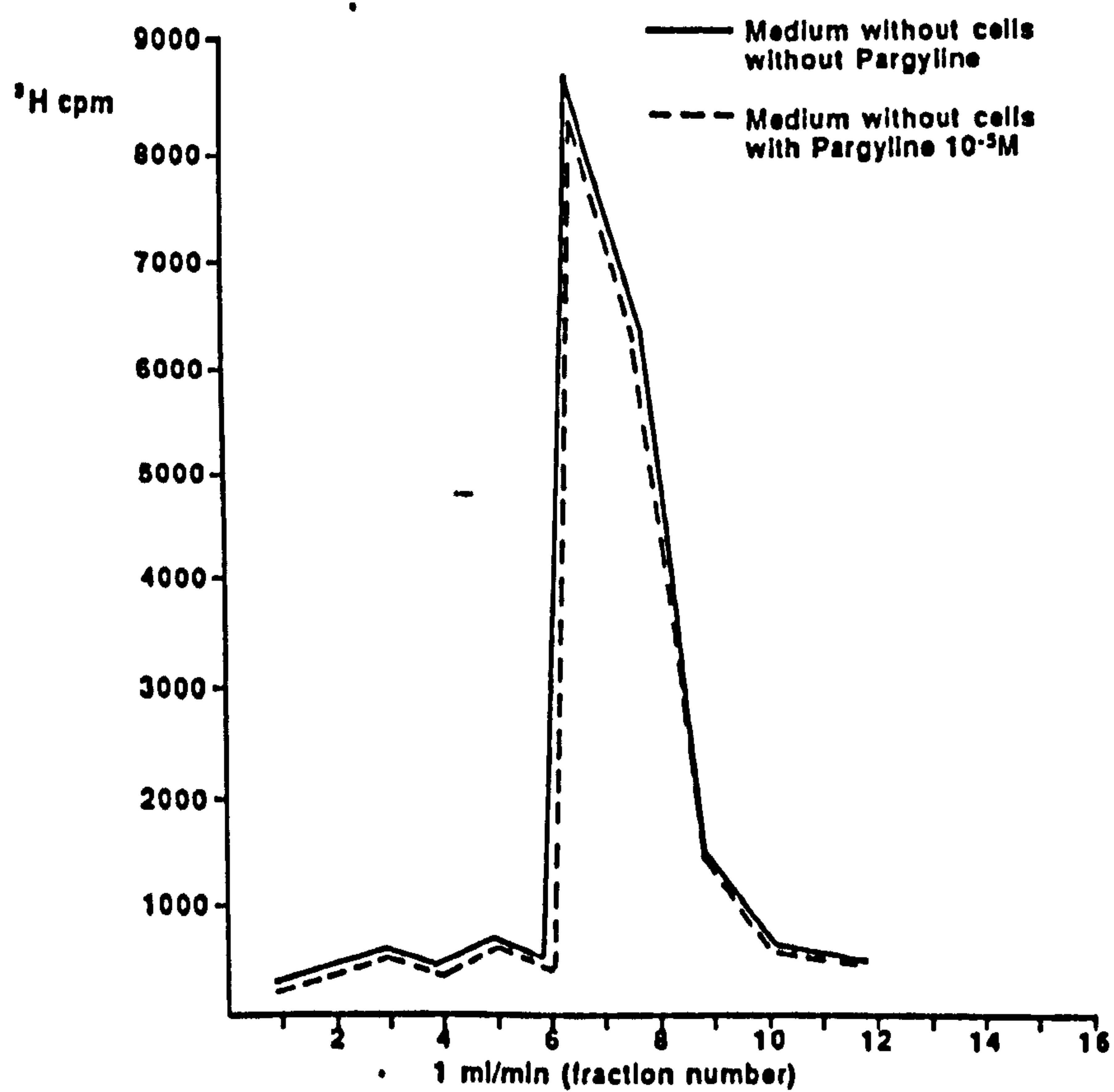


Fig 9.8b

Profile of ^3H counts in supernatant of incubates without cells \pm Pargyline



Mobile phase 1ml/min

R 5HT = 6.4 mins

R 5HIAA = 4.46 mins

R *N*- α -methytryptamine (Internal std.) = 9.3 mins

Fig 9.9a

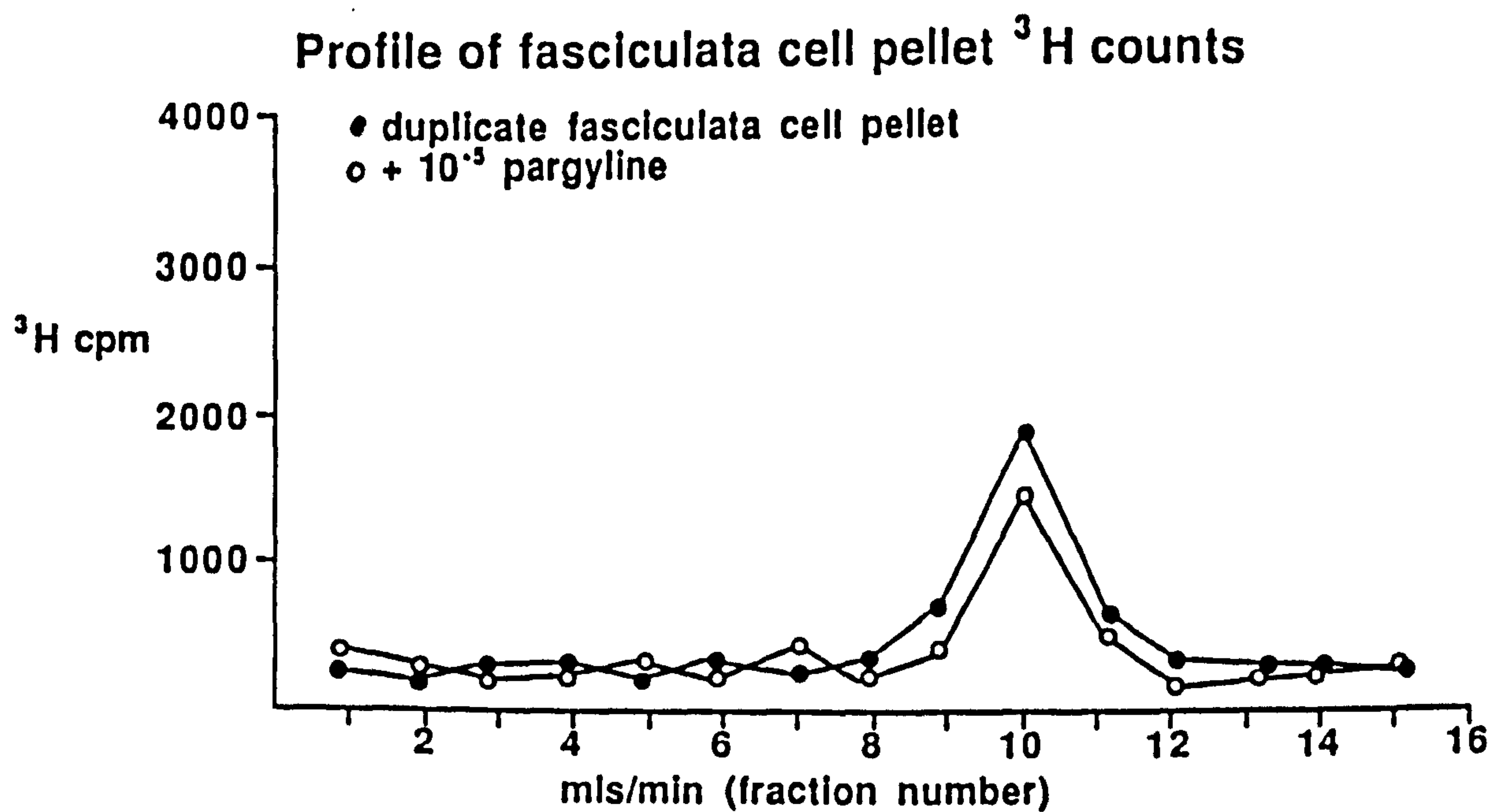
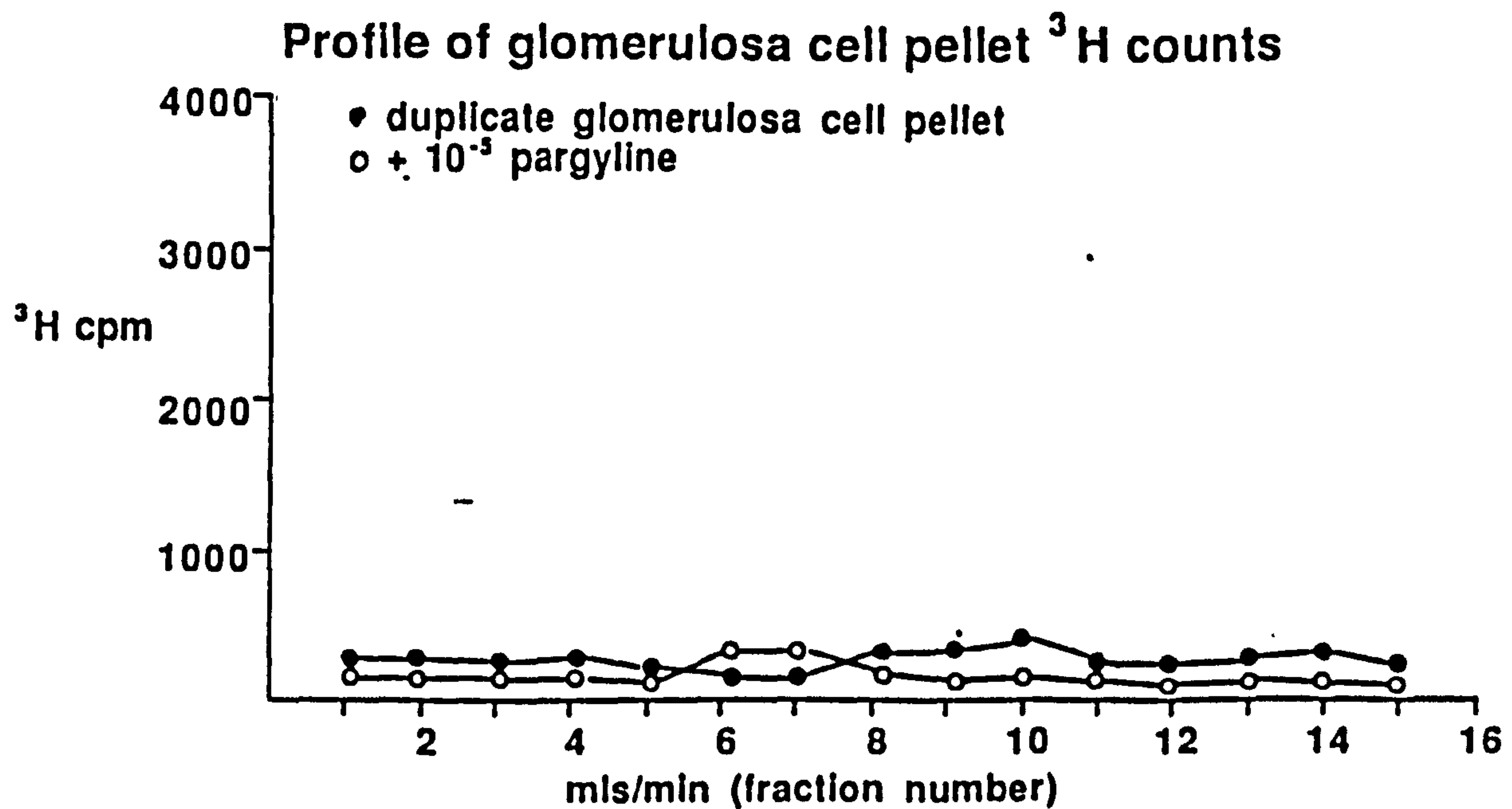


Fig 9.9b



Mobile phase 1ml/min

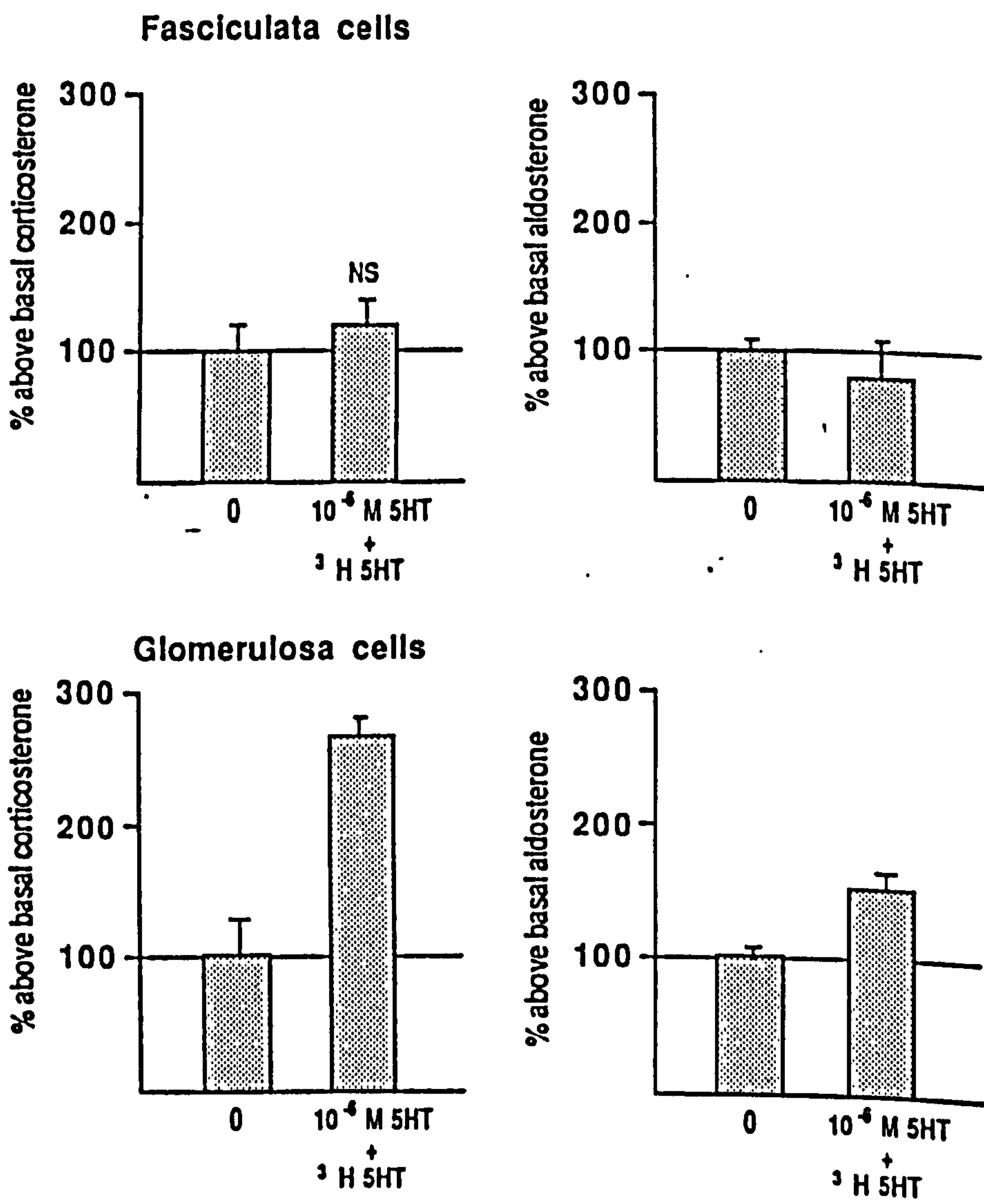
R_T 5HT = 8.5 mins

R_T 5HIAA = 5.4 mins

R_T Nmethyltryptamine (internal std.) = 10.8 mins

Fig 9.10

**Steroid output in glomerulosa and fasciculata cells
 $\pm 10^{-6}$ M 5HT and 3 H 5HT
(first experiment)**



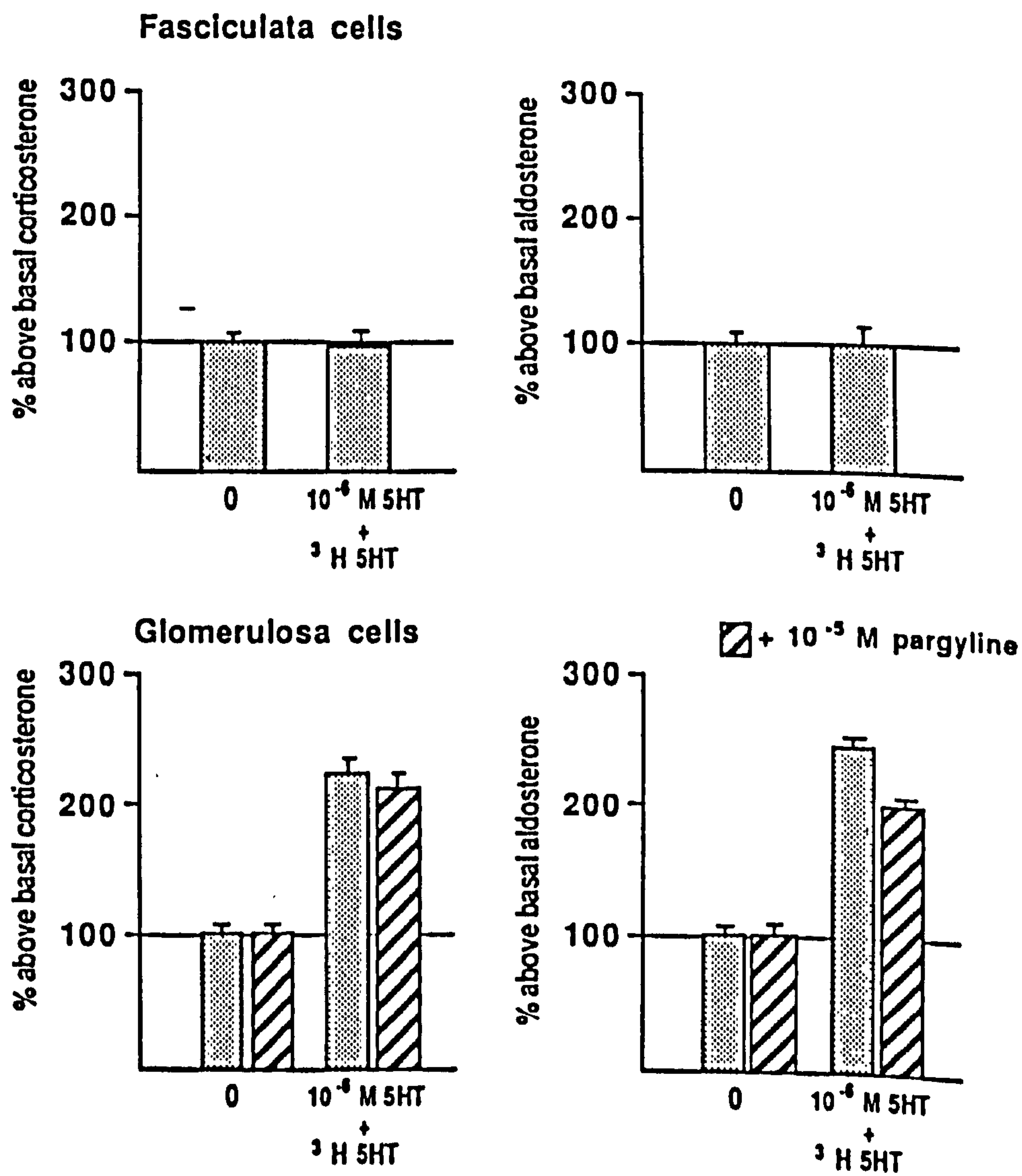
In experiment 1 the steroid output (Fig. 9.10) was also measured in both cell preparations. The inner zones showed no significant increase in either corticosterone or aldosterone in the presence of 5HT; zona glomerulosa cells responded to 5HT with a significant increase in aldosterone. In the second experiment (Fig.9.11), again, only the zona glomerulosa cells responded to 5HT; pargyline did not affect either cell type in increasing steroidogenesis, indeed there was a slight but significant depression in the stimulation of aldosterone in the presence of pargyline compared to control.

9.5 DISCUSSION

Serotonin metabolism is only partially inhibited by the monoamine oxidase inhibitor. This suggests several things: the inhibitor is not potent enough to inhibit all the MAO present at this dose (10⁻⁵M); or that the inhibitor is non-specific to Type A and Type B monoamine oxidase, so that at least some of the activity of one enzyme subtype still remains; Type A is present, since this specifically oxidizes 5HT, norepinephrine and epinephrine and 5HT is converted to 5HIAA in these studies. Type A monoamine oxidase is present specifically in intraneural locations and is found in sympathetic nerve endings. However, the lack of complete inhibition of 5HT metabolism, using pargyline, could also indicate the presence of Type B within the adrenal, the extra-neural subtype - particularly in the inner regions. Possibly of more importance, is the greater percentage use of 5HT and its larger concentration present in the

Fig 9.11

**Steroid output in glomerulosa and fasciculata cells
 $\pm 10^{-6}$ M 5HT and 3 H 5HT
(second experiment)**



cell pellet of the fasciculata cells and contaminating medullary tissue, compared to the glomerulosa cells; there is however an anomaly in the amount of total 5HT used in the inner zone (+ pargyline), compared to lower amounts present in the control cell pellet (- pargyline) - this could be due to indiscriminate 'leakage' of 5HT from the washed cell pellet. However, 5HT can be stored or remain unmetabolised in the inner zones to a greater extent overall as shown in the pooled results, 'with/without pargyline, compared to the glomerulosa cells. There remains the possibility that 5HT is taken up by medullary chromaffin granules possibly present in the inner cell preparation, or contaminating mast cells and platelets obviously present in larger amounts in the vascularized inner zones compared to the capsular zone. Livett et al., 1984, found that medullary rat adrenal tissue could be isolated and prepared in a similar procedure as employed for capsular cell preparations, although medullary tissue is more readily dispersed compared to cortical tissue. Thus it may be present, albeit in smaller quantity, in the cortical preparation. In the preparation of isolated capsular and decapsulated cells in the rat, Matsouka et al., 1981b, referred to the medullary cell contamination within the decapsular cell preparation which was also prepared by collagenase dispersal. Winkler et al., 1986 described specific amine carrier systems present within isolated adrenal chromaffin granules, which are capable of taking up exogenous 5HT as previously reported by Bertler in in vivo rabbit studies, and by Potter and Axelrod in

centrifugal sucrose density gradient isolation of labelled 5HT in chromaffin granules. Another possibility is that platelet contamination, particularly in the highly vascular inner zones, allowed for 5HT uptake into platelet uptake sites. Brain, medullary and platelet tissue all share a common mode of uptake for 5HT as well as other biogenic amines. Medullary tissue is modified sympathetic neuronal tissue and together with sympathetic nerve fibre endings, which have already been described in sheep and rat adrenal inner cortex (Robinson et al., 1977., Kleitman et al., 1985), may account for the greater apparent storage of 5HT within this region. Of course the reasons remain speculative, since further work would be needed to clarify these findings, including the isolation of medullary cells and the measurement of monoamine oxidase activity independent of the adrenal cortex. The important point to note however, is that the metabolism of 5HT, although nonspecific to the whole adrenal, is more marked in the glomerulosa zone where 5HT stimulates steroidogenesis; metabolism and/or storage are not the important factors in determining steroidogenic potency - this is also borne out in the indoleamine studies where 5HIAA did not stimulate aldosterone secretion. Thus, this rather suggests a specific, receptor mediated response, particular to the outer zona glomerulosa.

The possibility exists of: 1) a neural innervatory component (possibly catecholaminergic as described above since 5HT nerve fibres were not visualised in the immunohistochemistry experiments) (see Chapter 11) existing in this region. Serotonin released from

platelets or mast cells (Osim et al., 1983., Green et al., 1962) possibly in the wake of a physiological stimuli such as stress, haemorrhage, shock... as 5HT release has already been noted, e.g. when TSH (thyroid stimulating hormone) stimulates the release of mast cell constituents including 5HT (Green et al., 1962), may lead to displacement of catecholamines bound to nerve ending vesicles and affect a response including the stimulation of aldosterone secretion; 2) alternatively 5HT could displace or be co-stored in medullary epinephrine containing granules, as Kuriyama (1984) described, within the inner zones. Thus, 5HT may play a subsidiary alternate neural and/or paracrine role during increased sympathetic activity. Excess and non-bound exogenous 5HT, as appears to exist in the zona glomerulosa, could then be metabolised by endogenous monoamine Type A enzyme within this region and/or type B in the inner regions. High levels of MAO Type A have been measured in whole adrenal tissue (Viveros et al., 1979) in adult rats, which would lend credence to this theory. However the lack of total inhibition of 5HT conversion to 5HIAA, particularly in the inner zones could also indicate different concentrations of this enzyme in the different regions or heterogeneous subtypes of MAO as indicated above. Of further interest were the effects of inhibiting 5HT metabolism on the steroidogenic response in the glomerulosa cells. As the graphs show, in the zona fasciculata preparation, 5HT had no effect on steroid output as expected, whereas in the zona glomerulosa cells 5HT stimulated aldosterone secretion. However, the presence of pargyline did not enhance the steroid response to exogenous 5HT or even increase the

basal steroid levels in the control incubates. This at first would tend to suggest that an increase in 5HT levels presented to the adrenal in the presence of a MAOI did not potentiate the adrenal response to 5HT as might be expected in a neural innervatory situation, for example, when MAOI is added to synapses there is a potentiation of the nerve impulses due to increasing the local concentrations of neurotransmitter. There also exists the possibility that the large dose of non radioactive 5HT would blanket any potentiating effects that pargyline may exert on depleting metabolism; further, in this static cell system , it may not have been sensitive enough to detect changes in the potentiating effects of non-metabolised 5HT, although statistically, the small percentage drop in 5HT metabolism may also have been responsible for the lack of potentiation. Overall, the inner zones store and utilise more 5HT than the zona glomerulosa but the metabolism itself is not involved in eliciting the specific steroidogenic response. Pargyline, a non-specific inhibitor of monoamine oxidases tends to be more potent in inhibiting the conversion of 5HT to 5HIAA in the zona glomerulosa, although this does not reach statistical significance in the limited number of studies carried out during this time; more data needs to be collected to determine if indeed both types of monoamine are present in all zones of the rat adrenal, and whether Type A predominates in the zona glomerulosa with considerable amounts of Type B also present in the inner zone. The results, far from being conclusive, point towards a local and immediate receptor-mediated response at the level of the zona glomerulosa with the inner zones

serving a storage, and possibly local paracrine role in regulating aldosterone secretion.

Chapter 10

THE EFFECTS OF SALT DIET ON PLASMA 5HT AND ADRENAL 5HT LEVELS AND
THE IN VITRO RESPONSIVENESS TO AII AND 5HT IN THE ZONA GLOMERULOSA

10.1.1 THE EFFECT OF SALT STATUS ON AII AND 5HT DOSE RESPONSE CURVES IN ISOLATED ZONA GLOMERULOSA CELLS AND ON PLASMA CONCENTRATIONS OF ALDOSTERONE, PRA AND SERUM 5HT LEVELS

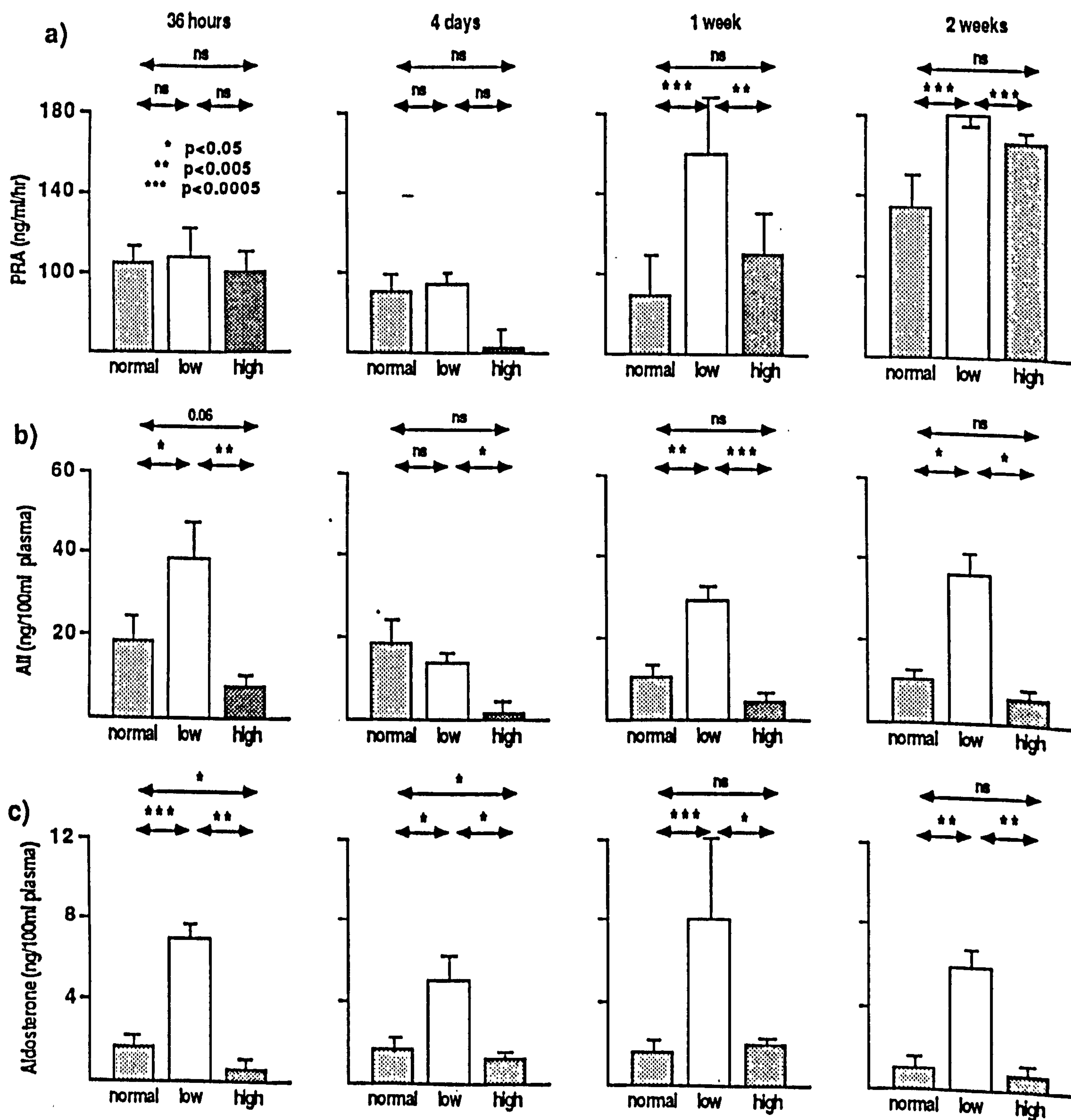
Serotonin may exert its steroidogenic effects through specific 5HT receptors that are subject to changes in sensitivity during altered sodium status. Aguilera demonstrated changes in AII receptor affinity and sensitivity, the latter extrapolated from a change in the magnitude of the steroid output obtained from rats on different sodium intake which paralleled changes in plasma aldosterone, AII and PRA levels. In the present studies the effects of a similar time study of altered sodium diets on the 'sensitivity' (as defined as a measure of the quantitative measure of the aldosterone response) of isolated zona glomerulosa cells to AII was studied to see if Aguilera's results could be reproduced, and further to determine whether the 5HT dose response curve was altered in any way with changing salt status. Serotonin levels in whole blood and in the adrenal gland were also measured to see if they altered as a consequence of a change in sodium intake.

10.1.2 METHODS

Female Wistar rats (100-150g) were placed in groups of six per cage and kept on wholemeal diets supplemented with 1% NaCl in the food in normal diets and water ad. libitum; low salt diet included wholemeal flour supplemented with 1% CaCO₃ and water ad. libitum; whilst high salt diet included 1% NaCl in the food and 1% saline for drinking.

Fig 10.1

Plasma aldosterone, PRA and All levels with normal, low and high salt diets
(time study)



(Fattah et al 1977). The rats were fed normal diets for a control 4 days before switching to the salt diet regimes for 36 hours, 4 days, 1 week and 2 weeks. After these time intervals the rats were sacrificed for in vitro incubations of zona glomerulosa cells or blood samples and whole adrenals collected for the measurements of plasma aldosterone, corticosterone, PRA, AII and 5HT in whole blood and tissue (see Chapter 6 for methods).

RESULTS

10.1.3 Plasma Measurements after 36 hours

After 36 hours PRA did not significantly change (Fig.10.1a) although plasma AII and aldosterone paralleled each other in accordance with salt status (Figs.10.1b and c). Thus, AII and aldosterone were significantly increased in low sodium status and decreased in high sodium status with respect to the control group. Whole blood 5HT levels were not significantly different in either low or high sodium status with respect to control groups nor was 5HT levels significantly different in the low salt state compared to the high group (Fig.10.2 and Table 10.1). The adrenal content of 5HT did not vary after 36 hours in both the decapsular and capsular portions of the adrenal glands (Tables 10.5 and 10.6).

10.1.4 Plasma Measurements after 4 days

PRA values were increased slightly but not significantly on low salt diets and depressed though not significantly on high salt diets compared to control group (Fig.10.1a). AII levels did tend to

Fig 10.2

Whole blood serotonin levels in normal, low and high salt diets:
changes with time

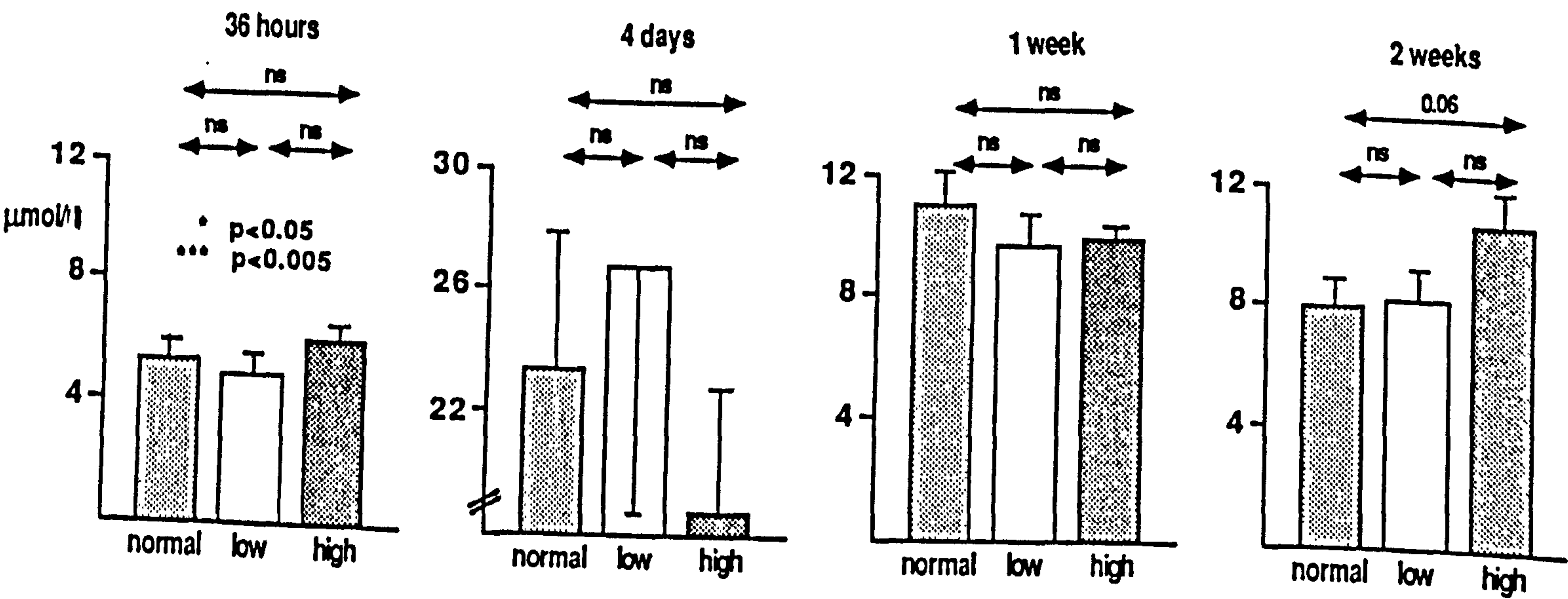
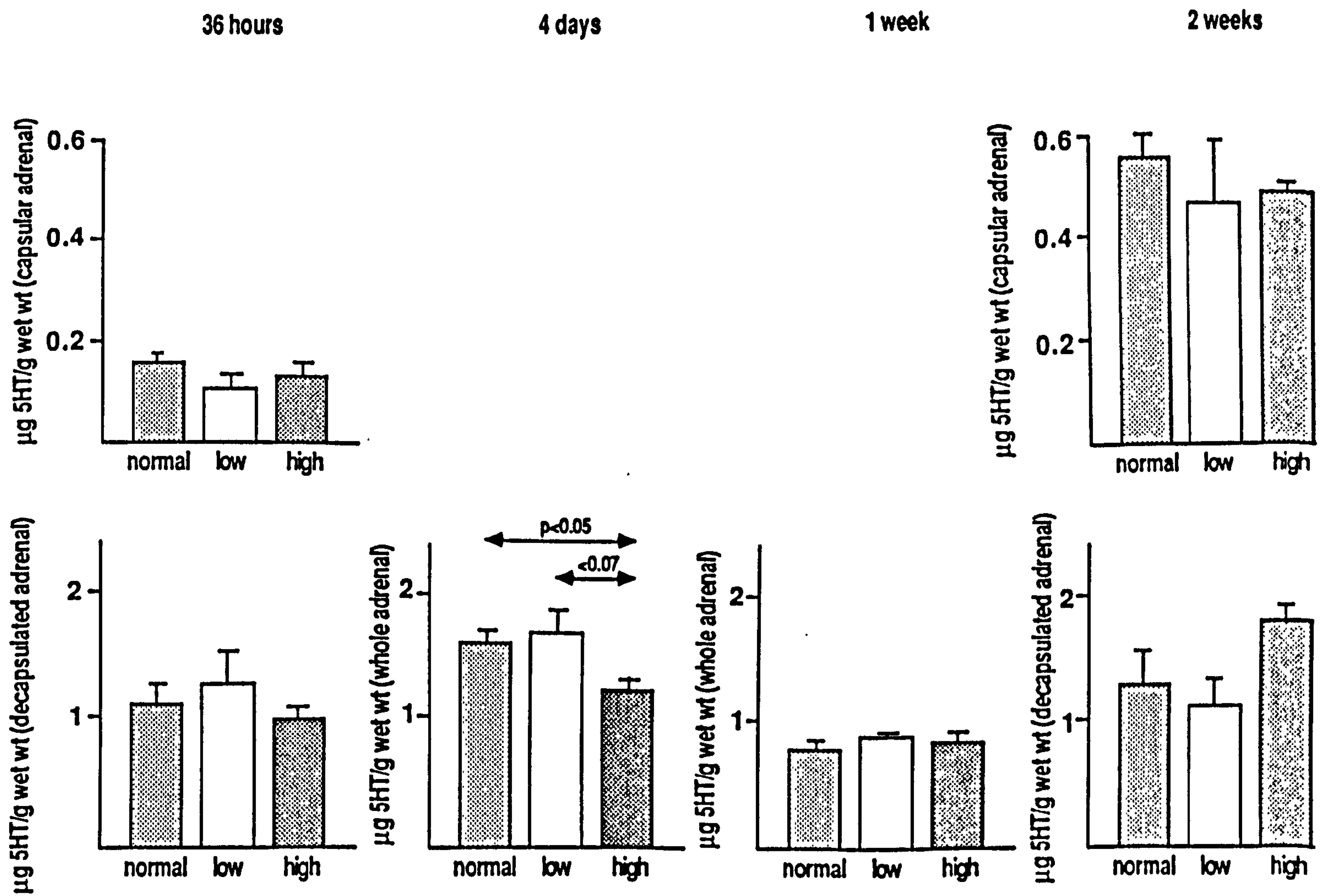


Fig 10.3

Serotonin levels in whole, capsular and decapsular adrenals on salt diets
(time study)



Whole Blood Serotonin Values for Normal, Low & High
Salt Diets : Time Study (mean \pm SD \pm SEM)

Table 10.1 (36 hours Na⁺ diets)

Normal μ moles 5HT/l	Low μ moles 5HT/l	High μ moles 5HT/l
5.0	2.6	5.7
4.3	6.1	5.7
7.2	4.0	7.8
6.6	3.2	6.4
4.3	6.25	6.4
5.2	6.6	6.6
(6)	(6)	(6)
5.4 \pm 1.1 \pm 0.5	4.8 \pm 1.5 \pm 0.7	6.2 \pm 0.8 \pm 0.36

(4 days Na⁺ diets)

Table 10.2

Normal μ moles 5HT/l	Low μ moles 5HT/l	High μ moles 5HT/l
15.4	41.2	6.7
40.8	37.8	13.6
30.0	34.0	42.7
6.4	2.5	12.5
15.8	-	7.3
(5)	(4)	(5)
21.7 \pm 13.6 \pm 6.1	28.9 \pm 17.8 \pm 8.9	16.6 \pm 14.9 \pm 6.7

Whole Blood Serotonin Values For Normal, Low & High
Salt Diets : Time Study (mean \pm SD \pm SEM)

(1 week Na⁺ diets)

Table 10.3

Normal μ moles 5HT/l	Low μ moles 5HT/l	High μ moles 5HT/l
11.4 6.0 11.9 14.7 (4)	8.3 9.35 1.3 8.5 (4)	9.1 10.6 10.8 9.6 (4)
11.0 \pm 3.1 \pm 1.7	9.8 \pm 1.8 \pm 0.6	10.0 \pm 0.7 \pm 0.4

(2 weeks Na⁺ diets)

Table 10.4

Normal μ moles 5HT/l	Low μ moles 5HT/l	High μ moles 5HT/l
8.0 8.2 11.3 5.4 5.4 9.6 (6)	8.5 6.5 6.0 6.1 10.3 11.8 (6)	11.0 9.6 8.6 16.4 10.2 10.5 (6)
7.9 \pm 2.1 \pm 0.9	8.2 \pm 2.2 \pm 1.0	11.0 \pm 2.5 \pm 1.11

SEROTONIN CONCENTRATIONS IN ADRENALS OF FEMALE
RATS ON NORMAL, LOW & HIGH SALT DIETS: TIME STUDY

CAPSULAR ADRENALS
(36 hours)

Table 10.5

Normal µg 5HT/g wet weight	Low µg 5HT/g wet weight	High µg 5HT/g wet weight
0.14 0.16 0.22 0.14 0.123 (5)	0.023 0.074 0.24 0.14 0.056 (5)	0.211 0.141 0.105 0.176 0.035 (5)
0.156 ± 0.03 ± 0.016	0.106 ± 0.07 ± 0.038	0.134 ± 0.06 ± 0.03

DECAPSULATED ADRENALS

Table 10.6

Normal µg 5HT/g wet weight	Low µg 5HT/g wet weight	High µg 5HT/g wet weight
1.65 1.1 0.95 1.02 0.93 - (5)	1.18 1.26 1.19 0.98 1.21 2.18 (6)	0.95 1.13 1.34 1.00 - - (4)
1.13 ± 0.26	1.33 ± 0.38	1.105 ± 0.5

parallel this change with no significant difference in low salt compared to the control 4 days study. On high salt, AII levels were significantly depressed relative to low salt ($p < 0.05$) but not different by statistical analysis (Students unpaired t-test) compared to normal salt status. However the large standard error of the mean was probably responsible for this result, although visually, there is a definite trend to decreased levels relative to the control group (Fig. 10.1b). Interestingly however, although PRA and AII levels are not significantly altered on low salt relative to normal salt status, plasma aldosterone shows significant changes according to salt status at this time interval (Fig.10.1c). This was paralleled by a similar trend in whole blood 5HT levels with respect to control values (Fig.10.2 and Table 10.2) although this did not reach statistical significance; a similar trend was seen in whole adrenal 5HT measurement although this was not statistically significant in the case of control vs low salt status (Fig.10.3 and Table 10.7).

10.1.5 Plasma Measurements after 1 week

PRA, AII and plasma aldosterone levels closely paralleled one another after 1 week salt diet changes (Fig. 10.1a,b and c). All parameters were significantly increased in low salt status relative to the control group and interestingly, high salt parameters were not significantly altered with respect to normal diets; they were however significantly lower in all cases, with respect to low salt status. This is in contrast to shorter time intervals in which high salt values are sometimes lowered with respect to the normal salt

Table 10.7

WHOLE ADRENALS
(4 day Na⁺ diet)

Normal µg[5HT] / g.wet. weight	Low µg[5HT] / g.wet weight	High µg[5HT] / g wet weight
1.51 1.44 1.88 1.58 - (4)	2.11 1.53 1.62 1.53 - (4)	1.09 1.51 1.18 1.34 1.18 (5)
1.6 ± 0.17 ± 0.09	1.69 ± 0.24 ± 0.14	1.26 ± 0.15 ± 0.07

Table 10.8

WHOLE ADRENALS
(1 week Na⁺ diet)

Normal µg[5HT] / g wet weight	Low µg[5HT] / g wet weight	High µg[5HT] / g wet weight
0.7 0.63 0.83 1.02 0.94 (5)	0.704 0.95 0.98 0.79 0.83 (5)	0.72 1.00 0.68 0.97 - (4)
0.82 ± 0.14 ± 0.07	0.85 ± 0.±0 ± 0.05	0.84 ± 0.14 ± 0.08

CAPSULAR ADRENALS
(2 weeks)

Table 10.9

Normal μg 5HT/ g wet weight	Low μg 5HT/g wet weight	High μg 5HT/g wet weight
0.45 0.74 0.56 0.45 (4)	0.422 0.45 0.53 0.74 0.26 (6)	0.67 0.56 0.405 0.211 0.65 (5)
$0.55 \pm 0.12 \pm 0.07$	$0.47 \pm 0.14 \pm$	$0.49 \pm 0.17 \pm 0.08$

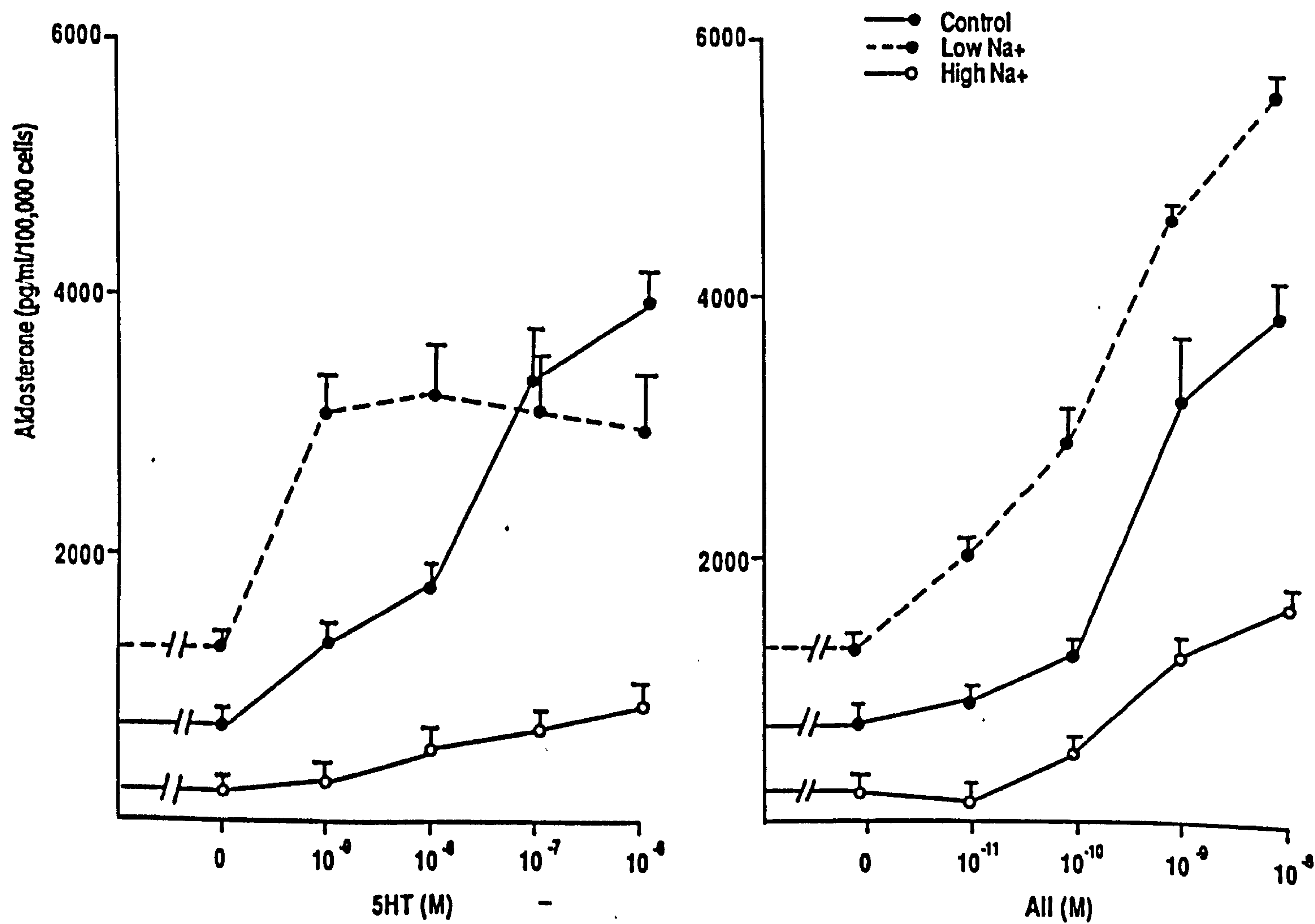
Table 10.10 **DECAPULATED ADRENALS**

Normal μg 5HT/ g wet weight	Low μg 5HT/g wet weight	High μg 5HT/g wet weight
1.04 2.64 0.77 0.84 0.98 1.33 (6)	1.72 0.57 0.67 1.48 1.62 0.72 (6)	1.95 0.70 1.57 2.3 1.3 2.86 (6)
$1.26 \pm 0.6 \pm 0.28$	$1.12 \pm 0.49 \pm 0.22$	$1.77 \pm 0.69 \pm 0.31$

() values in parenthesis indicate number of rats in study.
Statistics by unpaired Students t - test $p < 0.05$ level.

Fig 10.4a

Aldosterone dose response curve to 5HT and All : 36 hour salt diet
1st Experiment



diet values. 5HT levels were not significantly different after this time in serum (Fig.10.2 and Table 10.3) or in the whole adrenal gland (Fig.10.3 and Table 10.8) in high or low salt status.

10.1.6 Plasma Measurements after 2 weeks

PRA, AII and aldosterone levels were significantly elevated in low sodium status with respect to control values but in the high salt state, they were not significantly different from control values (Fig. 10.1a,b and c). Whole blood 5HT levels were not significantly different in the low salt group compared to control values, and were just above the $p < 0.05$ level of significance in the high salt group compared to the control group (see Fig.10.2 and Table 10.4).

Serotonin levels were not significantly different in any of the groups in the capsular and decapsulated portions of the adrenal gland (Fig.10.3 and Tables 10.9 and 10.10).

10.2 IN VITRO RESULTS OF SALT DIETS

10.2.1 Dose Response Curves to 5HT and AII (36 hours)

In the first 36 hour experiment (Fig.10.4a) the basal levels of aldosterone in vitro were altered by salt diet. The steroid output and sensitivity, as defined as a change in steroid output at a given dose, was increased for the dose response curve to AII and 5HT on low salt diet and the maximal output was increased for AII alone.

Conversely, high salt diet reduced steroid output at all doses of 5HT and AII. Point to point the steepness of the response curve was increased at the lowest dose of AII and 5HT on low salt, compared to

control; in high salt diet, the steepness was reduced at the lowest dose of AII and 5HT compared to control. The results were plotted as a percentage above basal, rather than percentage of the maximal response, since a plateauing of the response was not always achieved, demonstrates an increase of 230% aldosterone output in low salt status at the lowest dose of 5HT used (10^{-9} M) compared to 180% output in normal and 100% on high salt diet. Looking at both this plot and Fig.10.4a showing absolute outputs, it can be seen that the initial slope of the dose response curves are not superimposable but rather, on low salt at 10^{-9} M 5HT, the sensitivity is increased relative to control and at the same lowest dose on high salt, the sensitivity is decreased. The magnitude of the absolute response is reduced markedly at all doses of 5HT but the percentage levels above basal (Fig. 10.4b) approach low salt percentage levels, even at 10^{-7} - 10^{-6} M 5HT. As can be deduced therefore from this result, salt status at 36 hours affects the sensitivity of the response at the lower levels of 5HT and in the case of high salt status, at all doses of 5HT, but does not affect the magnitude of the response in absolute terms in low salt compared to control at higher doses of 5HT (10^{-7} - 10^{-6} M). For the AII dose response to changing salt, the steroid output was markedly elevated at all doses of AII in low salt relative to normal salt diets, and only at the lower doses of AII, was the magnitude of the response, both in absolute terms and in percentage terms, greater with respect to the control group (Fig.10.4a and 10.4c). In high salt all doses of AII did not elicit as great an absolute response as

Fig 10.4b
% above basal aldosterone for 5HT stimulation : 36 hour salt diet
1st Experiment

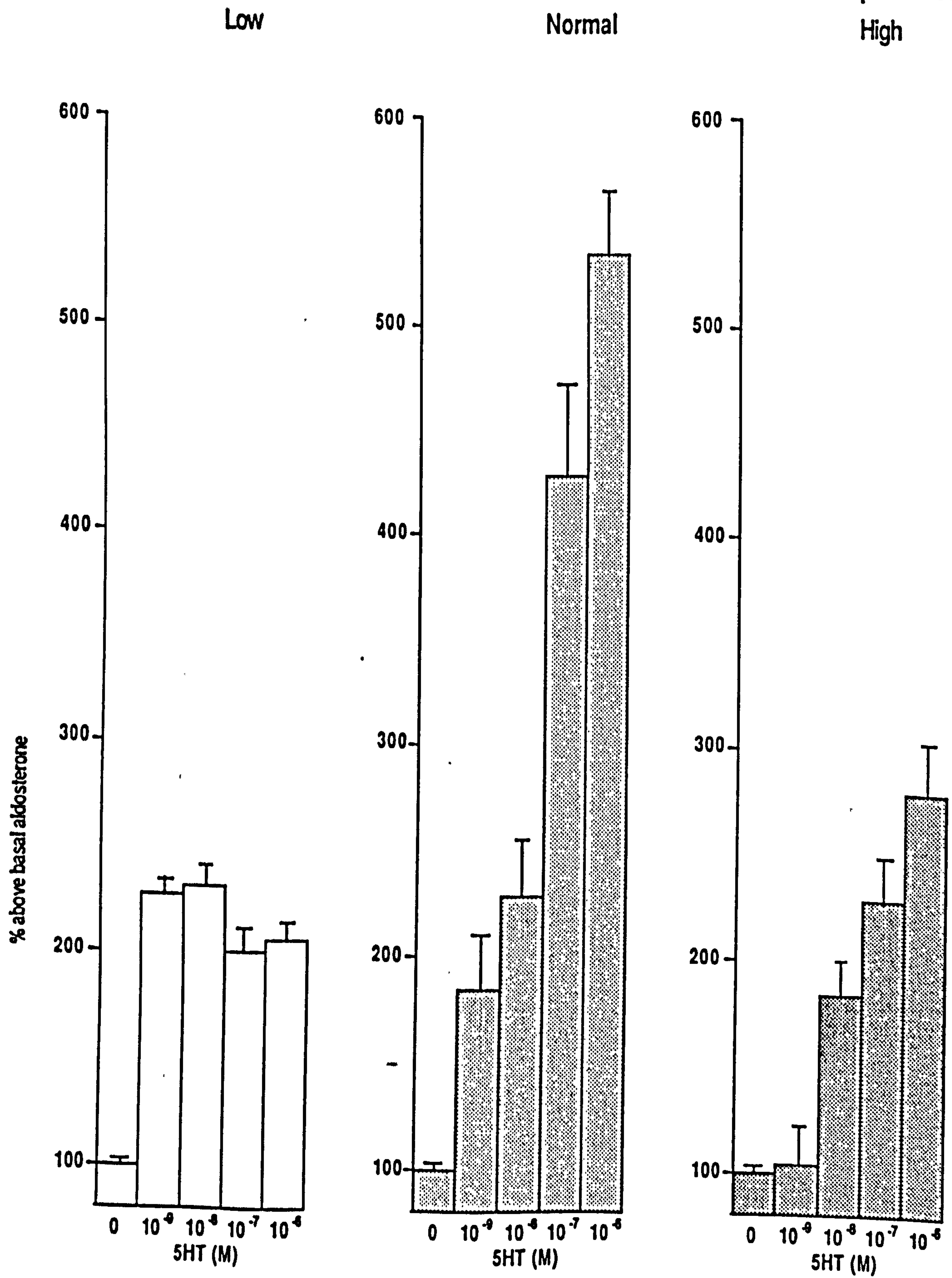


Fig 10.4c

% above basal aldosterone for All stimulation : 36 hour salt diet
1st Experiment

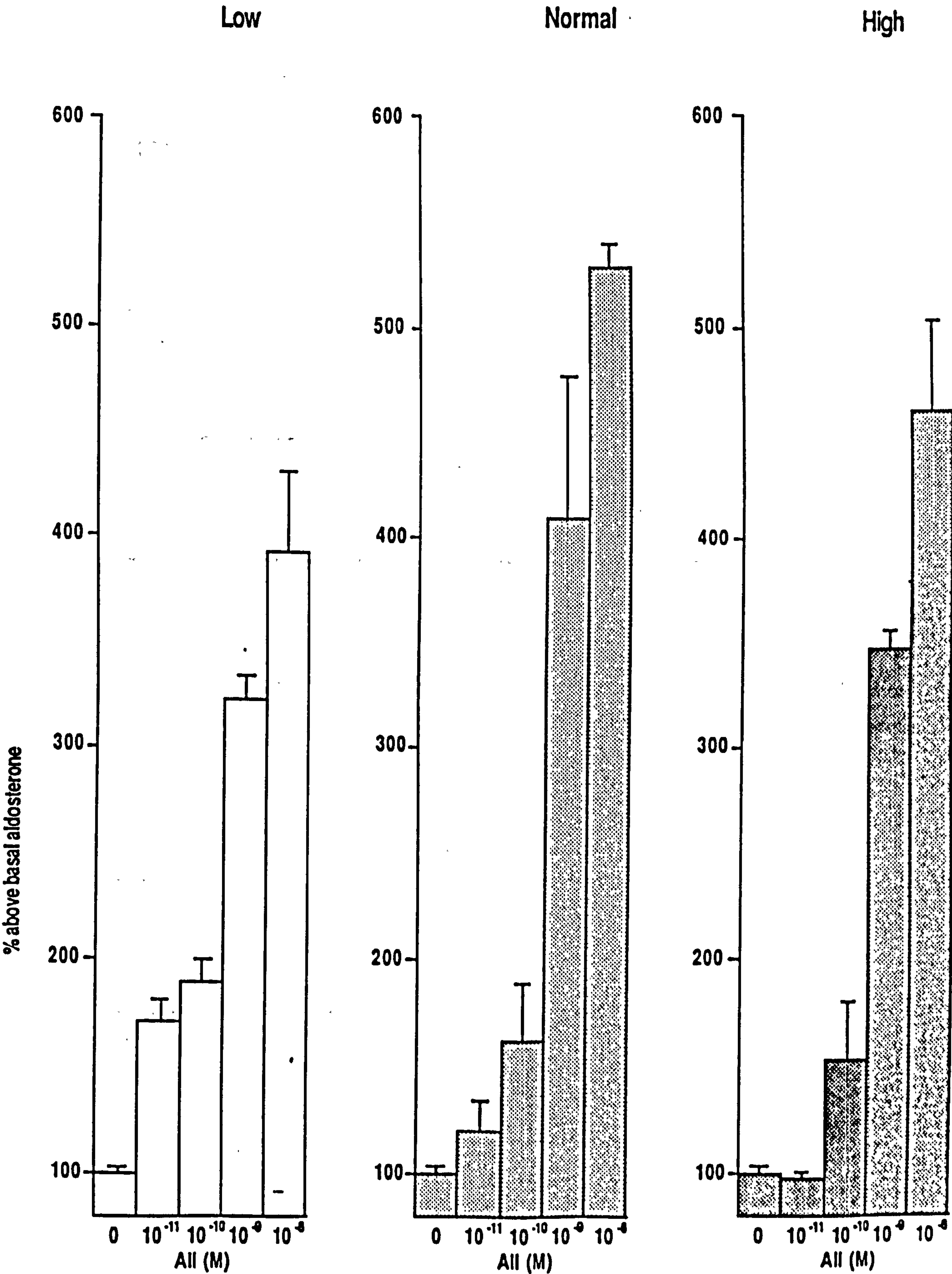


Table 10.11
p-values for % above basal aldosterone
: 36 hours salt diet (1st experiment)

Stimulus (M)

5HT

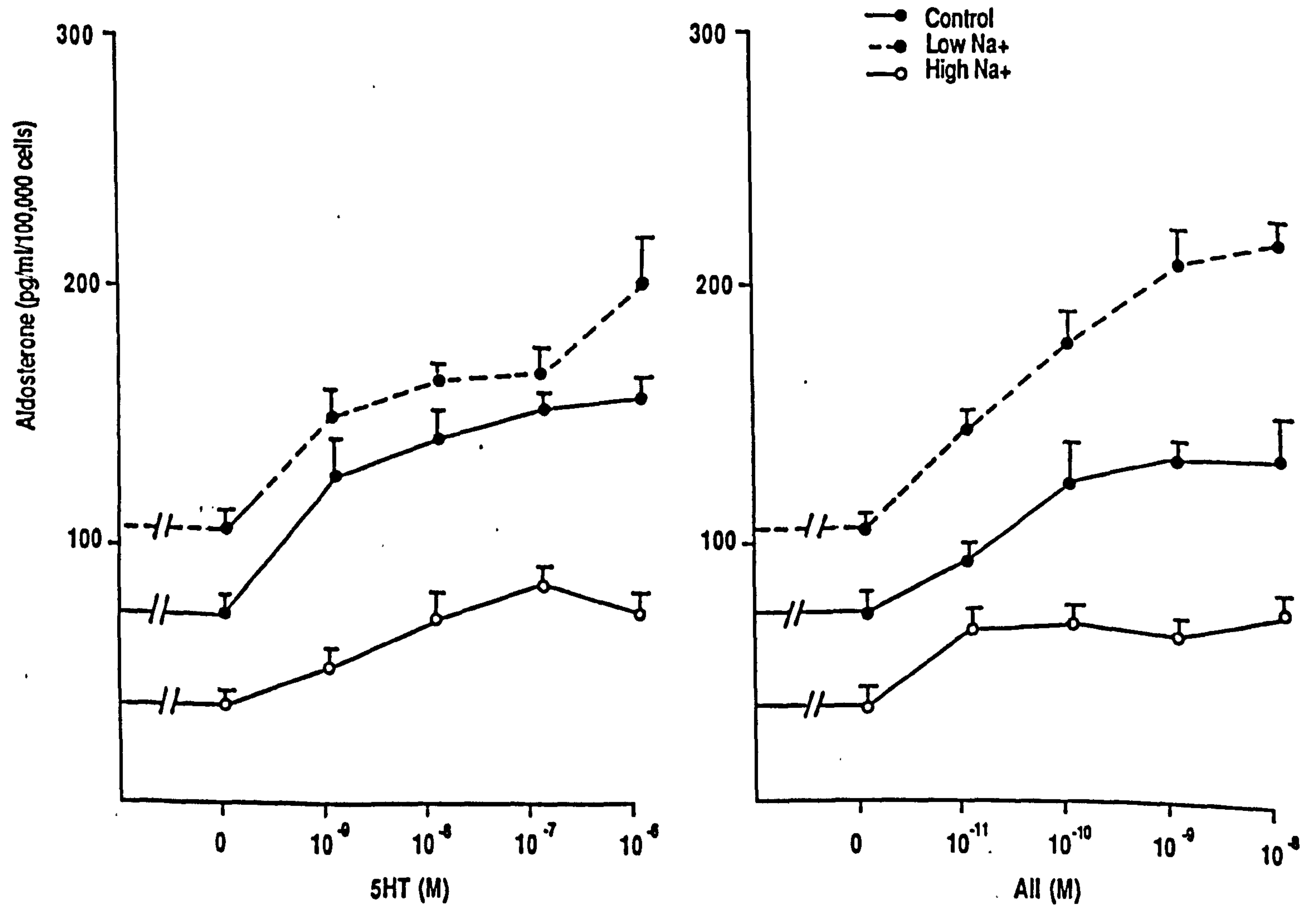
10 ⁻⁹	Normal/Low Normal/High Low/High	NS 0.05 0.003
10 ⁻⁸	Normal/Low Normal/High Low/High	NS NS 0.05
10 ⁻⁷	Normal/Low Normal/High Low/High	0.002 0.002 NS
10 ⁻⁶	Normal/Low Normal/High Low/High	0.0002 0.005 0.05

All

10 ⁻¹¹	Normal/Low Normal/High Low/High	0.005 NS 0.004
10 ⁻¹⁰	Normal/Low Normal/High Low/High	NS NS NS
10 ⁻⁹	Normal/Low Normal/High Low/High	NS NS NS
10 ⁻⁸	Normal/Low Normal/High Low/High	0.05 0.05 NS

Fig 10.5a

Aldosterone dose response curve to 5HT and All : 36 hour salt diet
2nd experiment



control when the curves were superimposed. In percentage terms (Fig. 10.4c) the sensitivity to AII was increased in low salt diet at 10^{-11} M AII whereas both in absolute terms and in percentage terms, there was no significant stimulation on high and control diets at 10^{-11} M AII. In the second experiment, (Fig.10.5a) basal steroid output was again altered by sodium status as was the steroid output point to point along the respective dose response curves. However, for the 5HT dose response curve, low salt status did not significantly alter the sensitivity of the response with respect to control when the curves were superimposed, i.e. they did not show competitive curve characteristics at any dose. Indeed, the percentage increases tended to be lower on low salt compared to the control curve (Fig. 10.5b) although this did not represent any significant difference (Table 10.12). In contrast, aldosterone output was decreased at all doses of 5HT on high salt compared to the control response, although by statistical analysis of percentage changes, this was not significantly depressed with respect to the control percentage values; only the responsiveness of the cells was changed by the salt regime. In this experiment the cells were all more responsive to AII on all salt regimes, with 10^{-11} M eliciting a steroid response. The magnitude of the responses to AII at lower doses of AII although in absolute terms varied with basal levels, upon superimposition, were not markedly different with respect to control, even in high salt diets, i.e. they were not competitive curves - indicative of a change in sensitivity, as defined as a change in ED_{50} by more

Fig 10.5b
% above basal aldosterone for 5HT stimulation : 36 hour salt diet
2nd experiment

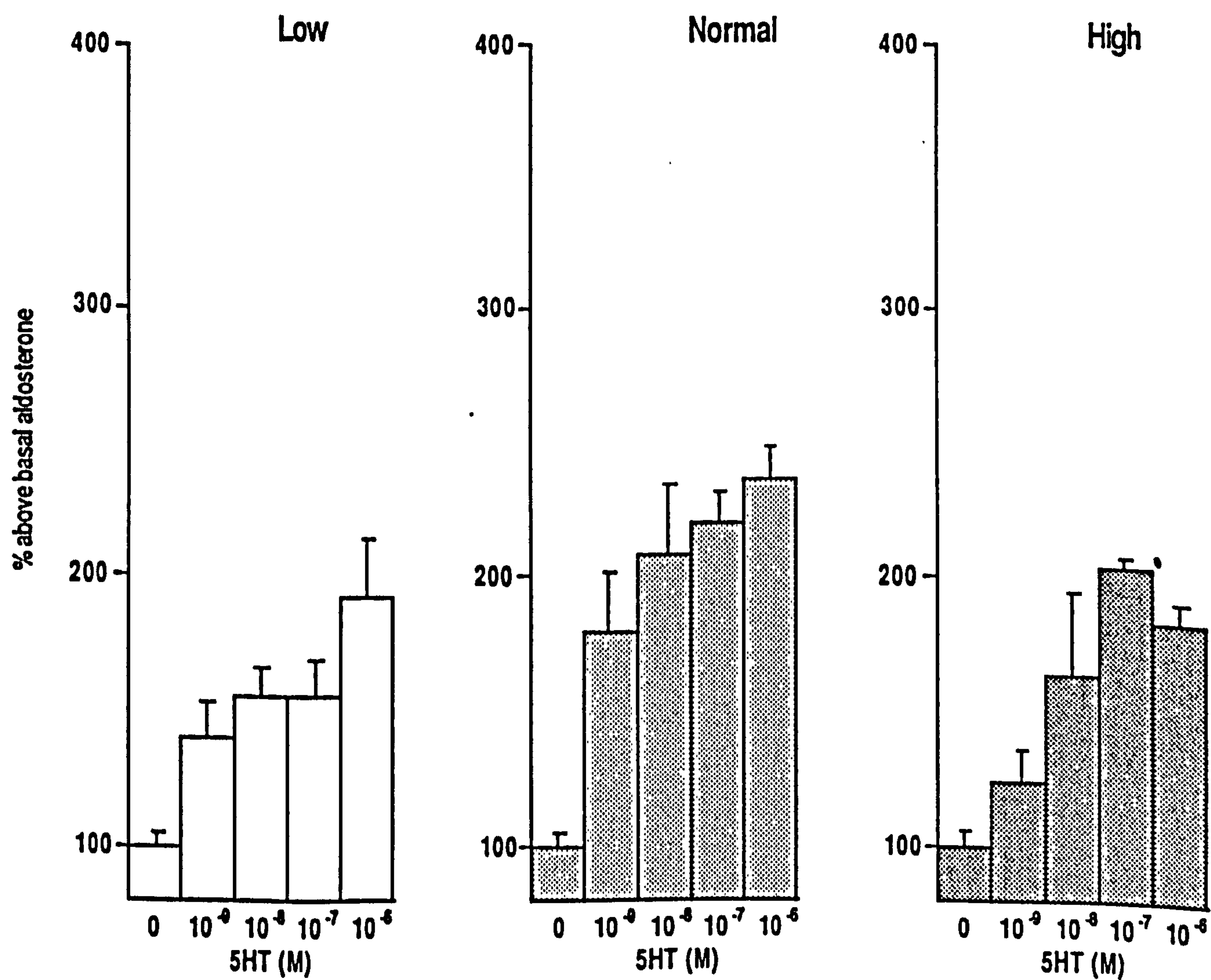


Fig 10.5c

**% above basal aldosterone for All stimulation : 36 hour salt diet
2nd Experiment**

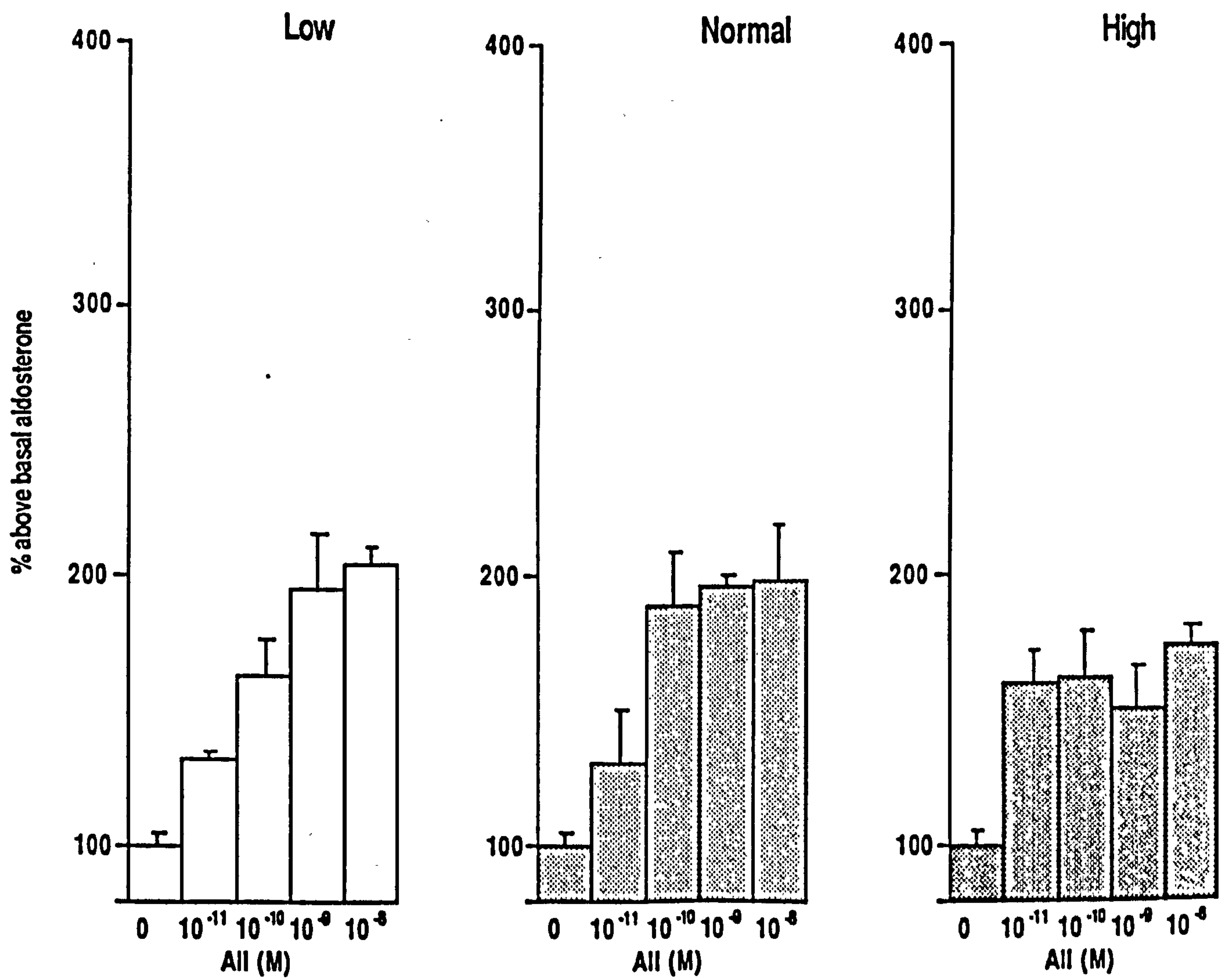


Table 10.12
p-values for % above basal aldosterone
: 36 hours salt diet (2nd experiment)

Stimulus (M)

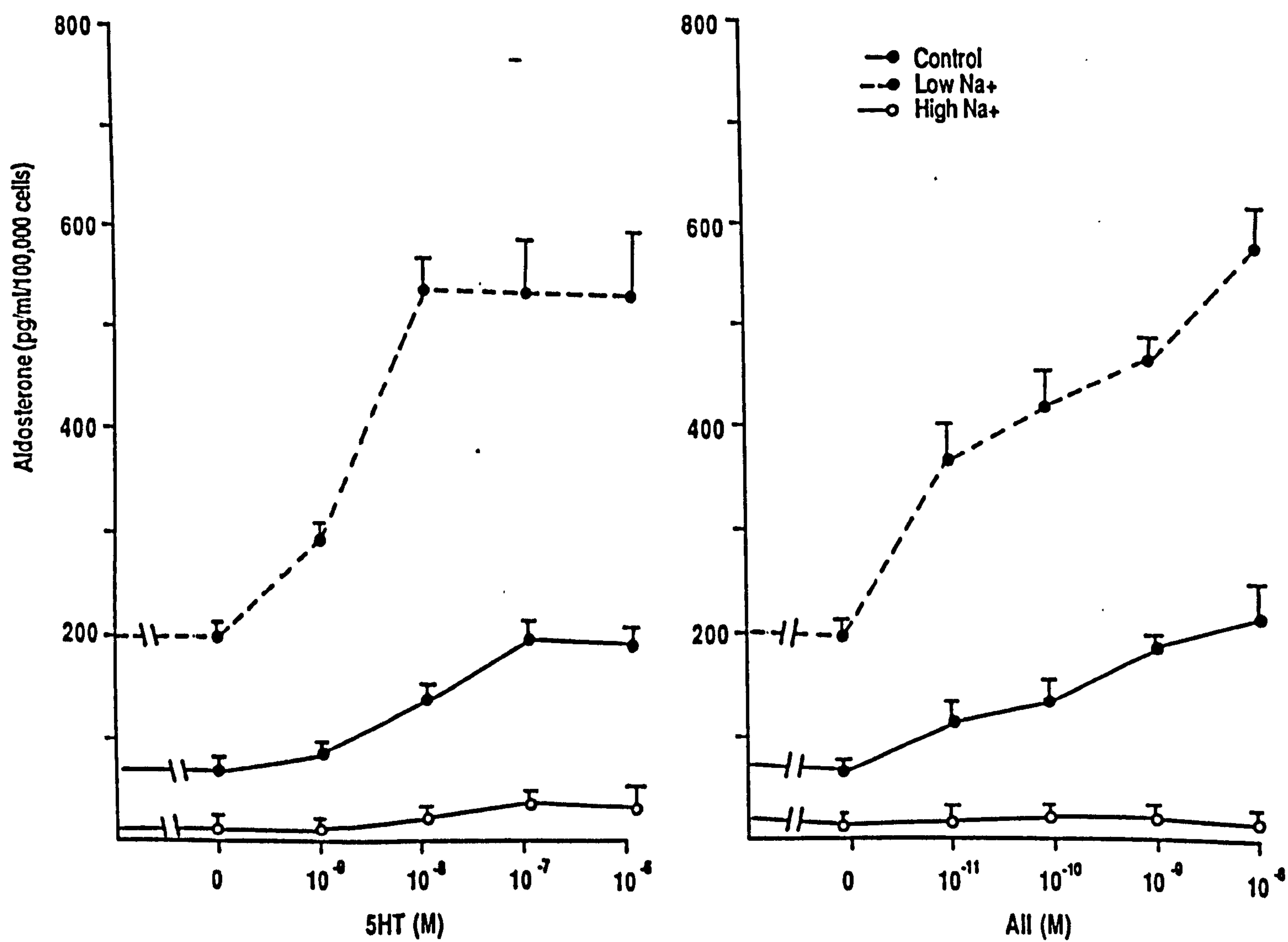
5HT

10 ⁻⁹	-	Normal/Low	NS
		Normal/High	NS
		Low/High	NS
10 ⁻⁸		Normal/Low	NS
		Normal/High	NS
		Low/High	0.07
10 ⁻⁷		Normal/Low	0.01
		Normal/High	NS
		Low/High	0.02
10 ⁻⁶		Normal/Low	NS
		Normal/High	0.01
		Low/High	NS

All

10 ⁻¹¹		Normal/Low	NS
		Normal/High	NS
		Low/High	NS
10 ⁻¹⁰		Normal/Low	NS
		Normal/High	NS
		Low/High	NS
10 ⁻⁹		Normal/Low	NS
		Normal/High	0.01
		Low/High	0.06
10 ⁻⁸		Normal/Low	NS
		Normal/High	NS
		Low/High	0.06

Fig 10.6 a
Aldosterone dose response curves to 5HT and All : 4day salt diet



rigorous curve-fitting computer analysis - this was not employed in these experimental studies. Only the absolute maximal output appeared to be altered by salt status. In both experiments the dose response to 5HT on high salt appeared to be attenuated more markedly than the dose response to AII on high salt.

10.2.2 Dose Response Curves to 5HT and AII (4 days)

Figure 10.6a shows a representative dose response curve to AII and 5HT in one out of two experiments and in both cases, the basal aldosterone levels were altered with respect to salt status as had been seen at 36 hours. Thus, on low salt diets basal levels were significantly greater than control values, and depressed with respect to control in high salt diets. The absolute responses are altered with respect to salt status for both stimuli at all doses. Serotonin stimulated steroidogenesis at the lowest dose 10^{-9} M 5HT on normal salt diet; the magnitude of the response in absolute terms was greater on low salt, thus indicating an increased sensitivity. However, on a percentage basis, this was not significantly different when compared to the control percentage increase (Fig. 10.6b and Table 10.13). Superimposing the high salt dose response curve to 5HT on the control curve, showed a reduced sensitivity as well as reduced responsiveness at all doses of 5HT-stimulated steroidogenesis. This was also reflected in reduced percentage increases of steroid output, particularly at lower doses of 5HT, which were significantly different compared to the control curve (Fig. 10.6b and Table 10.13). The increased responsiveness of the 5HT response in low salt,

Fig 10.6 b

% above basal aldosterone for 5HT stimulation : 4 days on salt diet

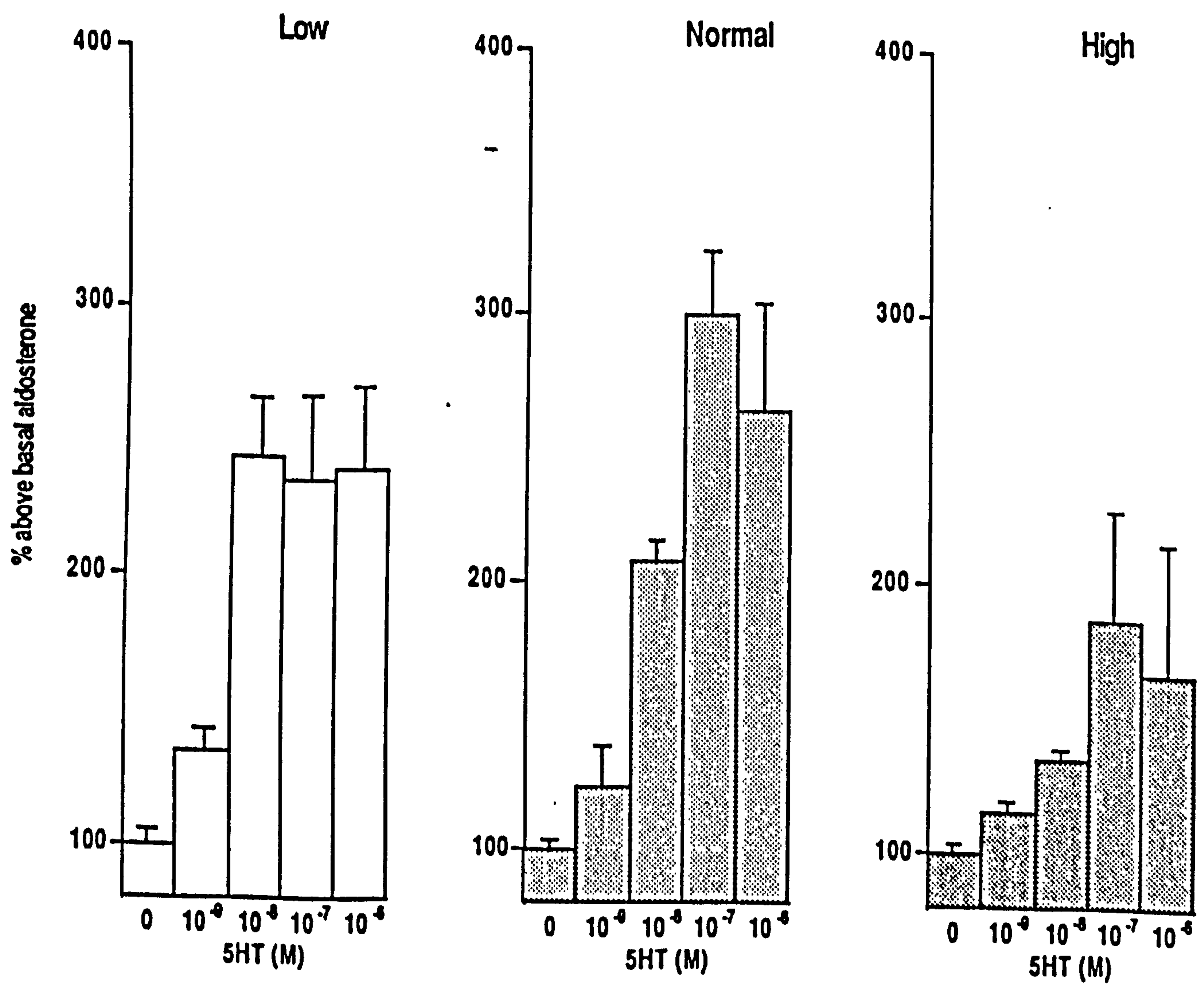


Fig 10.6c

% above basal aldosterone for All stimulation : 4 day salt diet

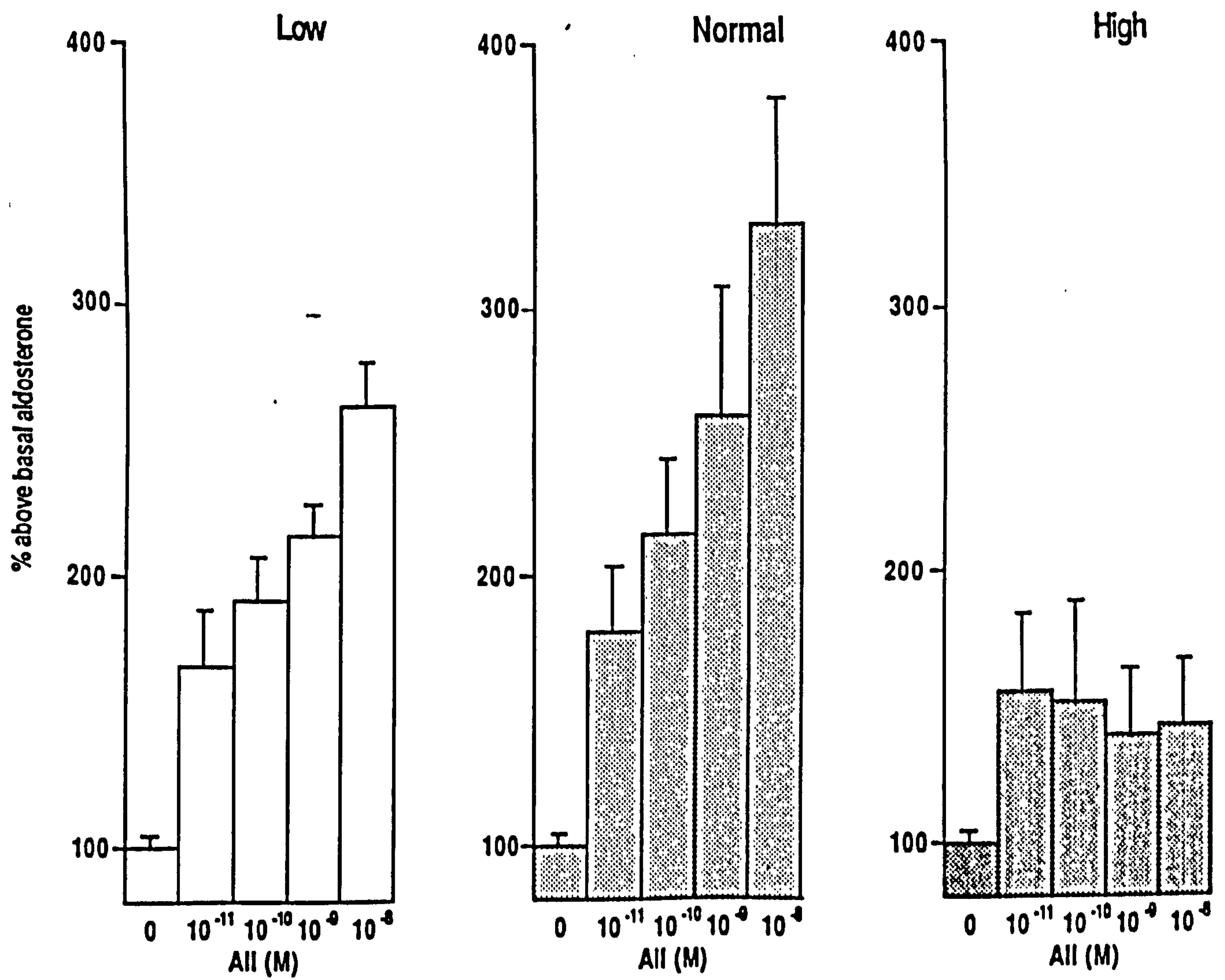


Table 10.13
p-values for % above basal aldosterone
: 4 days salt diet

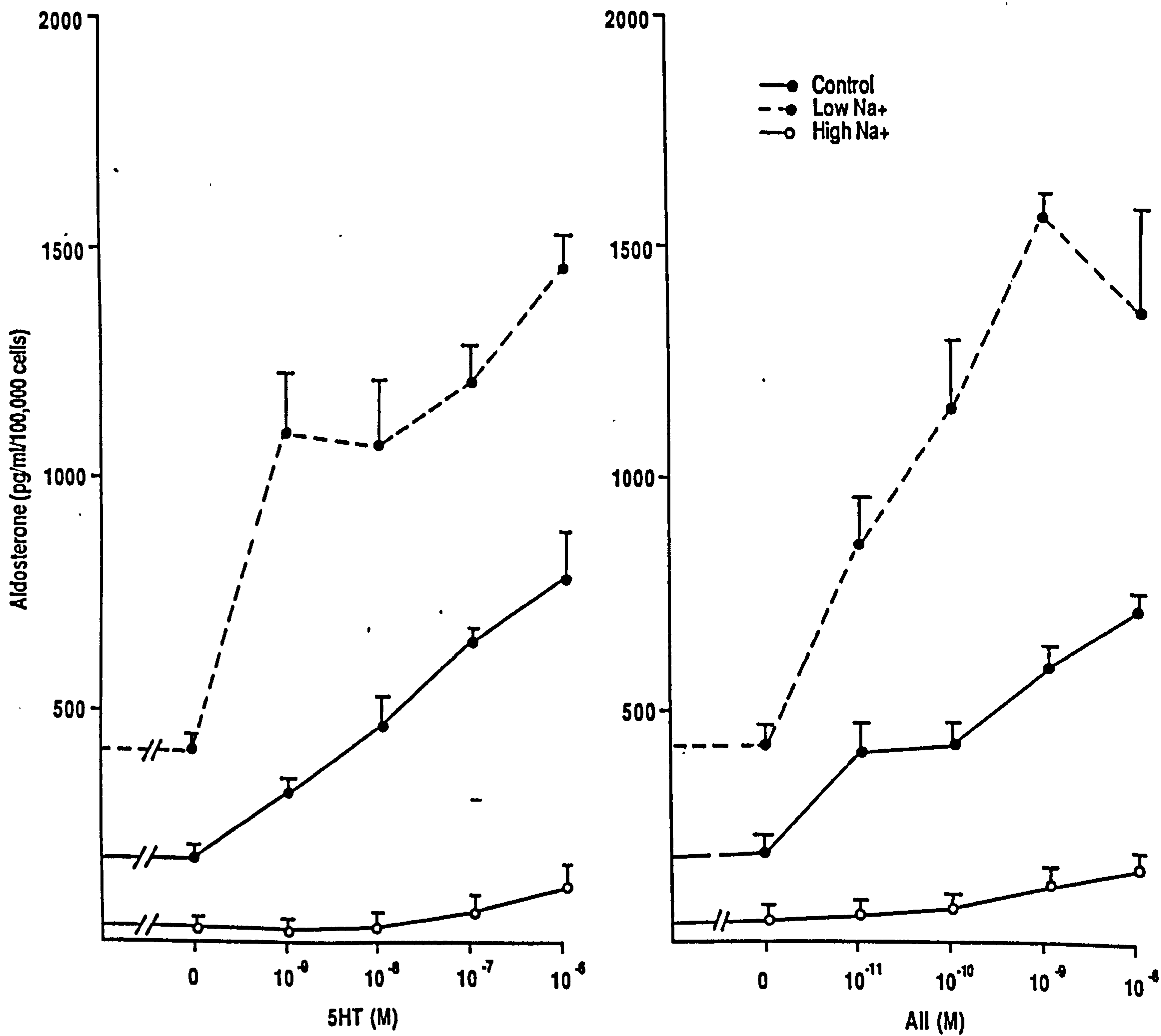
Stimulus (M)
5HT

10 ⁻⁹	Normal/Low Normal/High Low/High	NS NS 0.006
10 ⁻⁸	Normal/Low Normal/High Low/High	NS 0.001 0.02
10 ⁻⁷	Normal/Low Normal/High Low/High	NS 0.07 NS
10 ⁻⁶	Normal/Low Normal/High Low/High	NS NS NS

All

10 ⁻¹¹	- Normal/Low Normal/High Low/High	NS NS NS
10 ⁻¹⁰	Normal/Low Normal/High Low/High	NS NS NS
10 ⁻⁹	Normal/Low Normal/High Low/High	NS 0.06 0.03
10 ⁻⁸	Normal/Low Normal/High Low/High	NS 0.04 0.06

Fig 10.7a
Aldosterone dose response curves to 5HT and All : 1 week salt diet
1st experiment



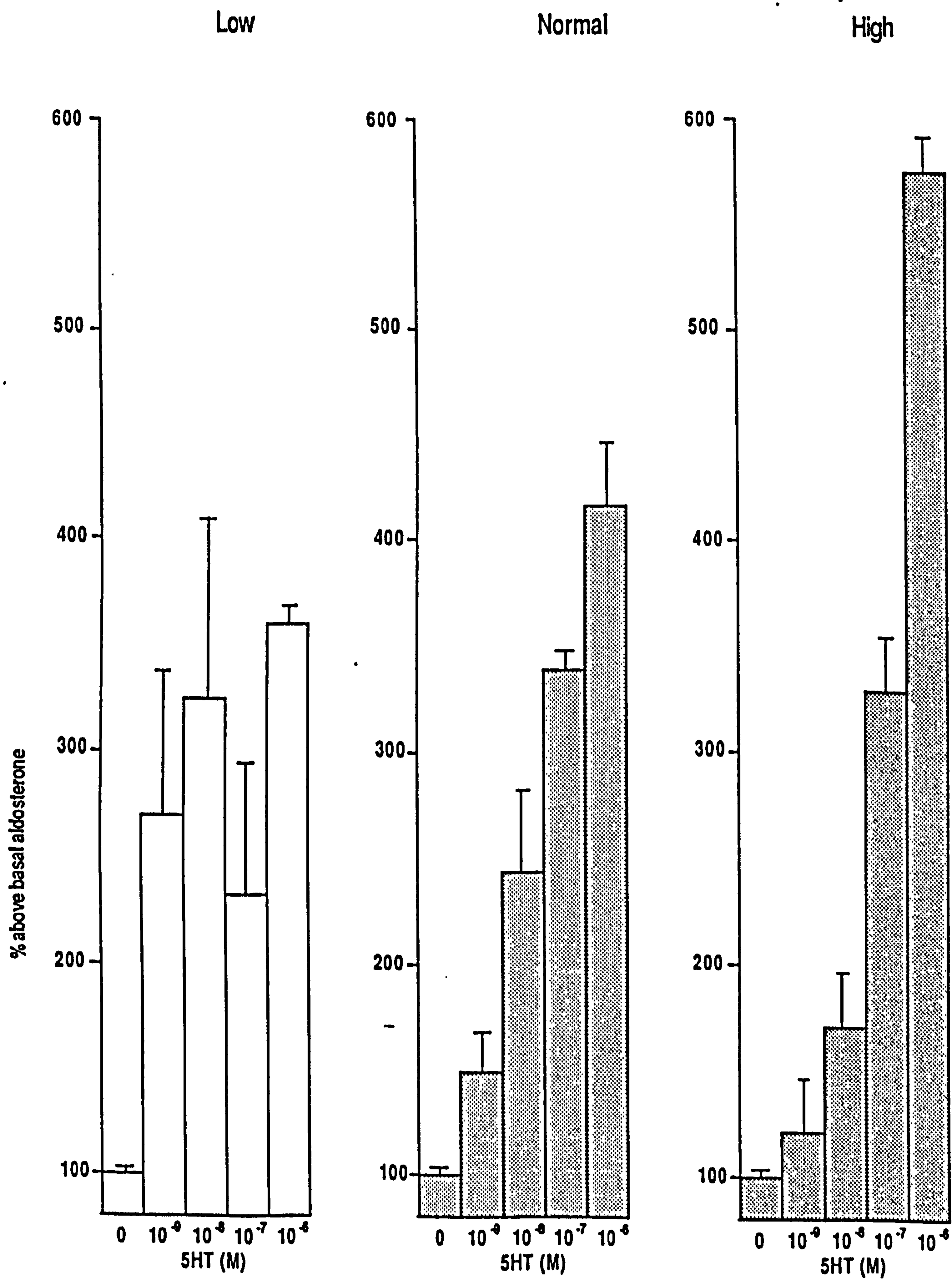
resulted in an earlier plateauing of the dose response compared to both normal and high salt diets i.e. at 10^{-8} M 5HT compared to 10^{-7} M 5HT on high and normal salt status.

The AII response curve changed in a similar fashion to the 5HT response curve although in this case, the sensitivity as defined in a change in the percentage increase at the lowest administered dose, did not alter even on high salt compared to control (Fig. 10.6c and Table 10.13) although here again, the responsiveness as measured in absolute terms was altered with respect to salt status.

10.2.3 Dose Response Curves to 5HT and AII (1 week)

In both experiments showing the dose response curves to 5HT and AII, basal aldosterone levels are significantly elevated on low salt diets and depressed on high salt diets compared to control values. In the first experiment (Fig.10.7a), the dose response curves for AII and 5HT on high salt were depressed at all points along the dose response curve compared to the control curve; conversely, the dose response curves were slightly steeper at some points along the dose response curve in low salt status and were slightly steeper at the lowest dose of 5HT and AII. However, superimposition of the dose response curves for both AII and 5HT in control salt diets onto low and high salt, showed that the maximal outputs altered in accordance with salt state, but that only the sensitivity to both stimuli was altered and reduced in high salt relative to the control response. In percentage terms (Fig. 10.7b) there was an increase in steroid output at 10^{-9} M 5HT relative to the same dose on different salt diets, which

Fig 10.7b
% above basal aldosterone for 5HT stimulation : 1week salt diet
1st Experiment



% above basal aldosterone for All stimulation : 1week salt diet
1st Experiment

High

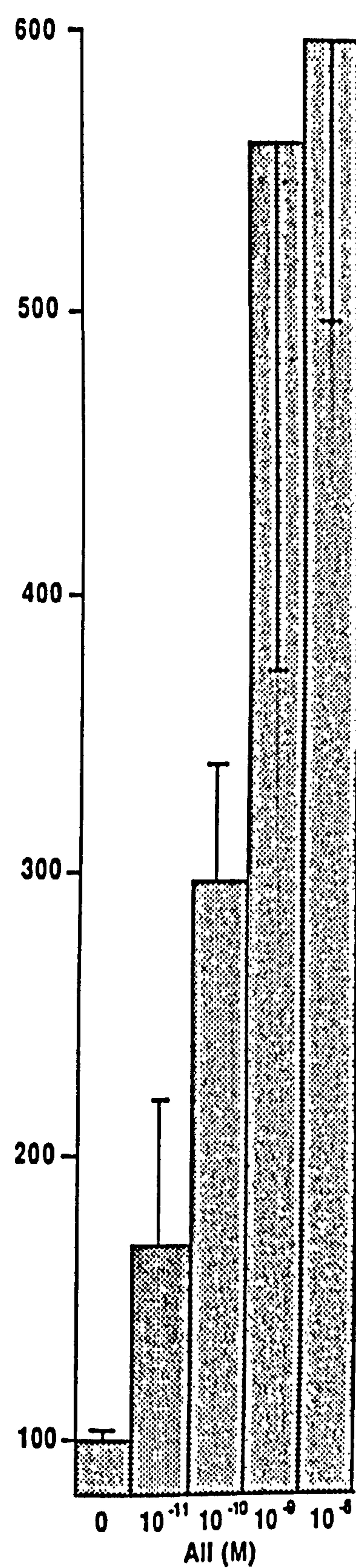


Table 10.14
p-values for % above basal aldosterone
: 1 week salt diet (1st experiment)

Stimulus (M)

5HT

10 ⁻⁹	Normal/Low Normal/High Low/High	0.05 NS 0.05
10 ⁻⁸	Normal/Low Normal/High Low/High	NS NS 0.05
10 ⁻⁷	Normal/Low Normal/High Low/High	0.05 NS NS
10 ⁻⁶	Normal/Low Normal/High Low/High	0.05 0.07 0.0002

All

10 ⁻¹¹	Normal/Low Normal/High Low/High	NS NS NS
10 ⁻¹⁰	Normal/Low Normal/High Low/High	NS NS NS
10 ⁻⁹	Normal/Low Normal/High Low/High	NS NS NS
10 ⁻⁸	Normal/Low Normal/High Low/High	NS NS 0.05

Fig 10.8a
Aldosterone dose response curve to 5HT and All : 1 week salt diet
2nd Experiment

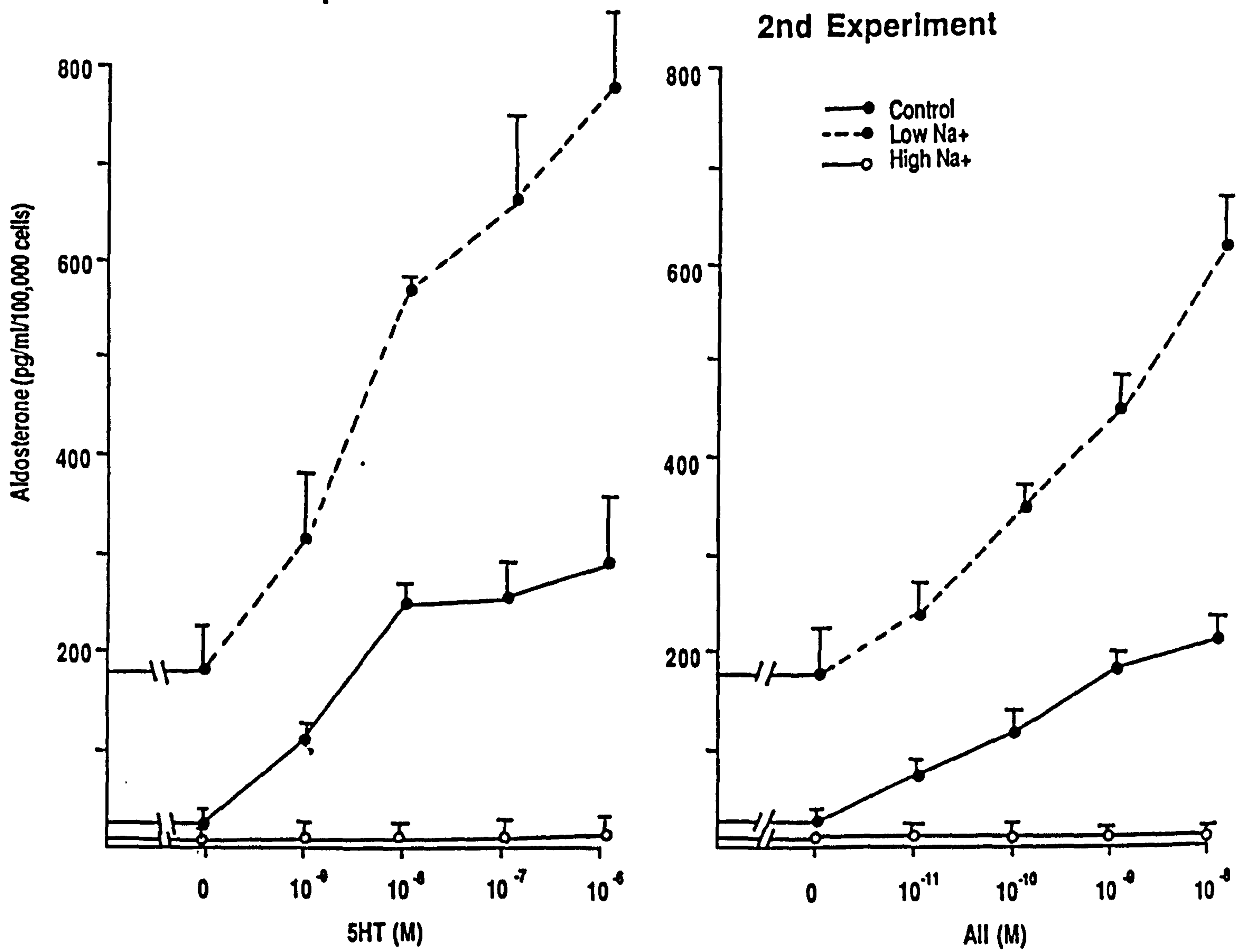


Fig 10.8b
% above basal aldosterone for 5HT stimulation : 1 week salt diet
2nd Experiment

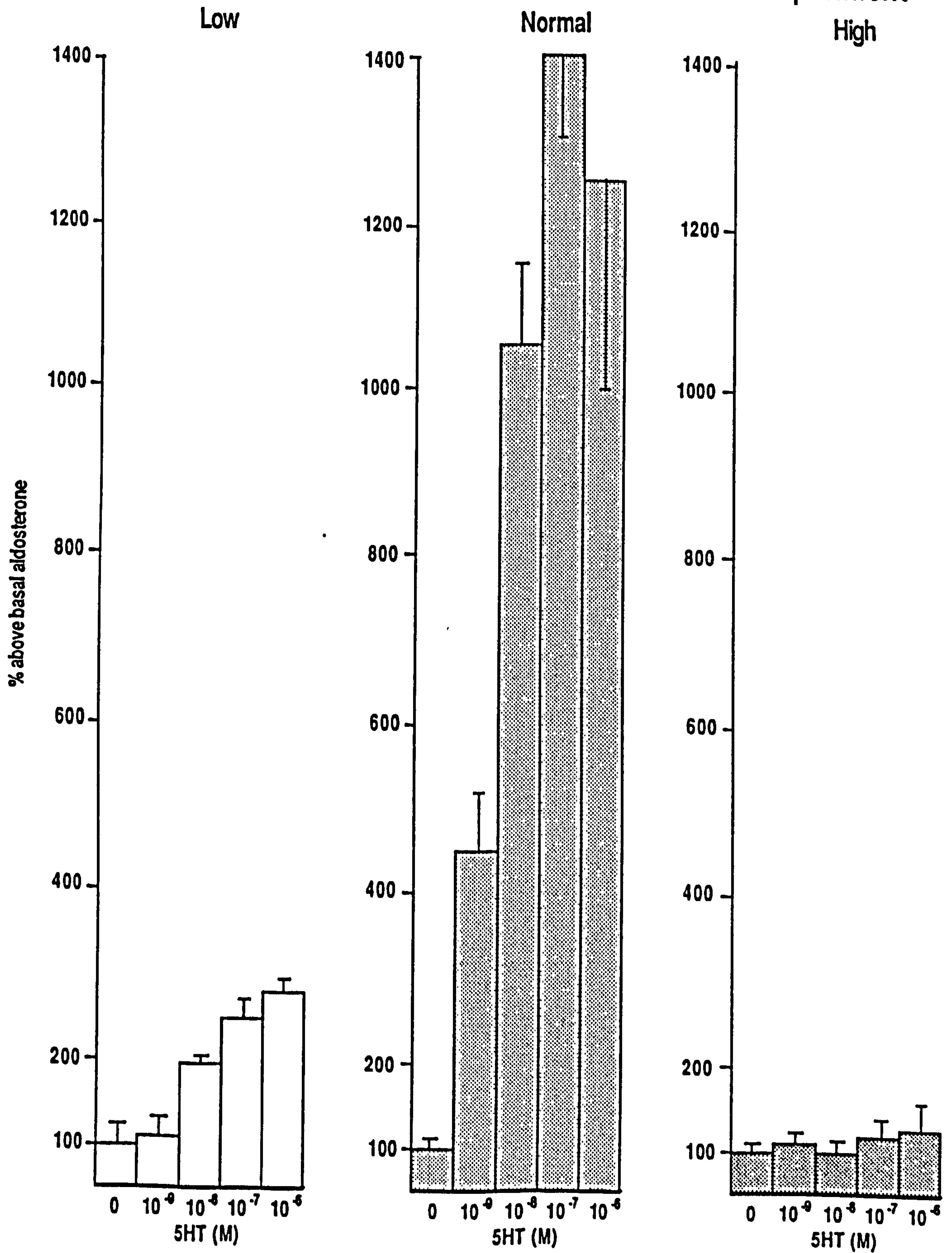


Fig 10.8c
% above basal aldosterone for 5HT stimulation : 1 week salt diet
2nd Experiment

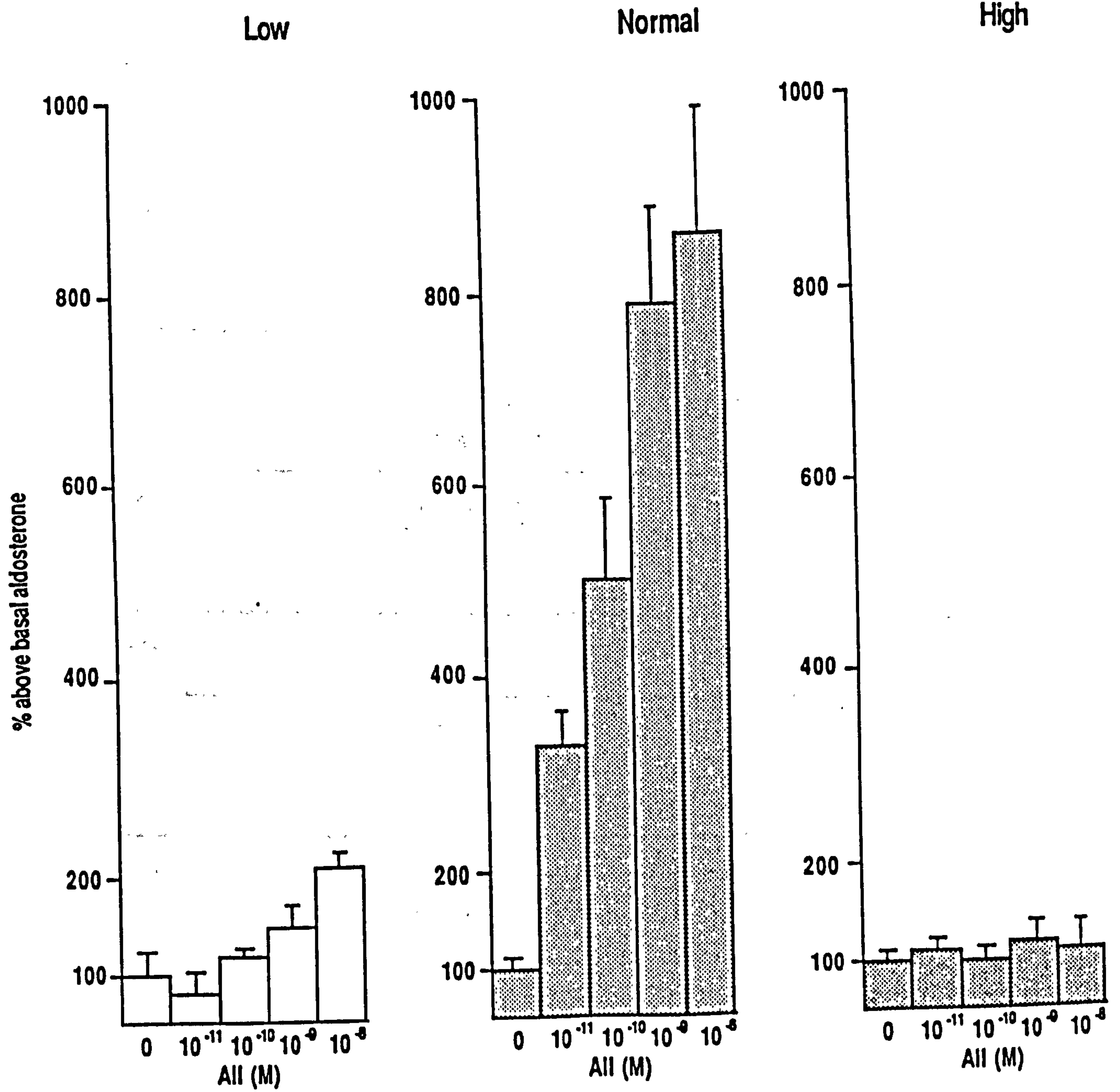


Table 10.15
p-values for % above basal aldosterone
: 1 week salt diet (2nd experiment)

Stimulus (M)
5HT

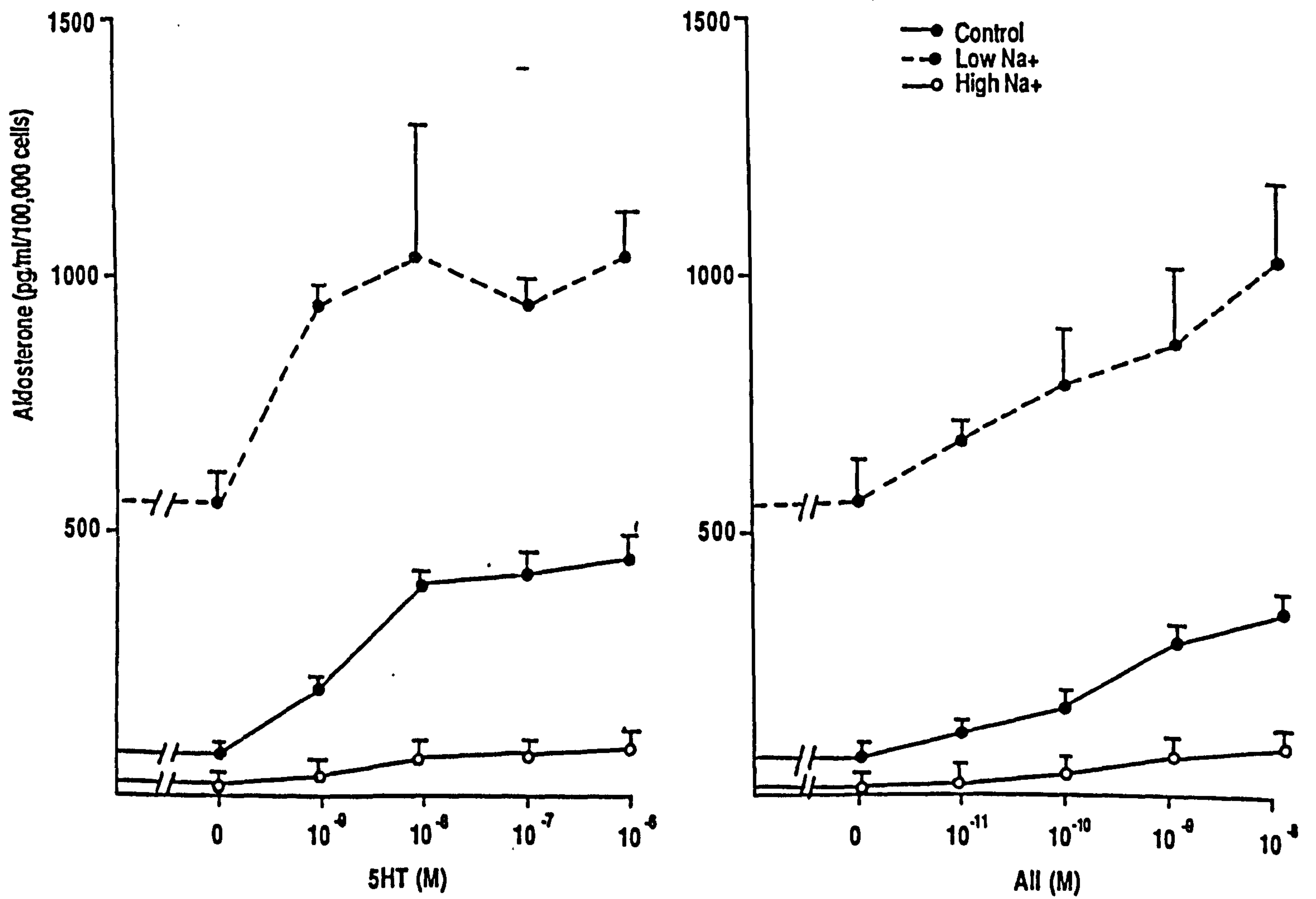
10 ⁻⁹	-	Normal/Low Normal/High Low/High	0.008 0.03 NS
10 ⁻⁸		Normal/Low Normal/High Low/High	0.01 0.01 0.0004
10 ⁻⁷		Normal/Low Normal/High Low/High	0.04 0.03 0.06
10 ⁻⁶		Normal/Low Normal/High Low/High	0.05 0.04 0.01

All

10 ⁻¹¹		Normal/Low Normal/High Low/High	0.001 0.02 NS
10 ⁻¹⁰		Normal/Low Normal/High Low/High	0.04 0.04 NS
10 ⁻⁹		Normal/Low Normal/High Low/High	0.02 0.02 0.07
10 ⁻⁸		Normal/Low Normal/High Low/High	0.05 0.04 0.01

was significantly different in low compared to control diets (Table 10.14); however, looking more closely, only on high salt diet was the response at the lowest dose likely to be non-significant and thus of reduced sensitivity compared to the other diet regimes. The same analysis holds for the AII response curves, with only high salt diet in absolute terms showing a reduced sensitivity (Fig.10.7c); the percentage values relative to each salt diet, particularly looking at the lower doses of AII, are not significantly different suggesting little change in responsiveness with respect to its own control. The second one week experiment (Fig.10.8a) is graphically similar to the first experiment (Fig. 10.7a) in that it shows a reproducible dose response to 5HT and AII. Superimposing the curves on each salt regime over the control response, indicates that the sensitivity to both AII and 5HT, is only affected and reduced in high salt compared to control; compounding this, the responsiveness with respect to its own control, is also depressed non-specifically in high salt for both AII- and 5HT-stimulated aldosterone production. In terms of percentage changes in both AII and 5HT-stimulated steroidogenesis in low and high salt diets, both are markedly affected and reduced compared with their control basal values (Figs.10.8b and c) and this shows up as a significant difference compared to the control percentage values (Table 10.15); conversely the high salt response in percentage terms is also significantly reduced with respect to the control percentage dose response curves (Table 10.15). The important factor which appears to determine the percentage above

Fig 10.9a
Aldosterone dose response curves to 5HT and All : 2 week salt diet
1st Experiment



basal values in each diet, is the variability in the basal measurement i.e. the low salt diet measure of steroid has a larger standard error bar compared to the control curve value. In this second experiment, the basal values for control and high salt are not significantly different and the shape of the respective dose response curves for AII and 5HT in each regime, indicate a compound change and reduction in sensitivity and responsiveness after 1 week high salt diet. Only the responsiveness is increased in the low salt regime.

10.2.4 Dose Response Curves to 5HT and AII (2 weeks)

In the first experiment (Fig. 10.9a) the basal values are altered in relation to the salt regime. For both AII and 5HT-stimulated steroidogenesis, there is stimulation at the lowest dose, on all regimes although this was not significant on high salt diets. However, as expected, the responsiveness is altered in parallel with a change in salt diet. Superimposing the curves in each regime for a given stimulus shows a change in responsiveness only for low salt compared to control, after 2 weeks; conversely, the sensitivity as a measure of the magnitude of the response is reduced slightly for high salt in both AII- and 5HT-stimulated aldosterone production. The anomalies arise in interpreting the results in percentage terms for as Figure 10.9b shows, the percentage increase relative to its own basal is reduced in the low salt compared to the normal dose response curve and this shows up as a significant difference with respect to control as shown in Table 10.16. The same holds true for the AII response curve; the high salt dose response curve compared to the

Fig 10.9b
% above basal aldosterone for 5HT stimulation : 2 weeks salt diet
1st Experiment

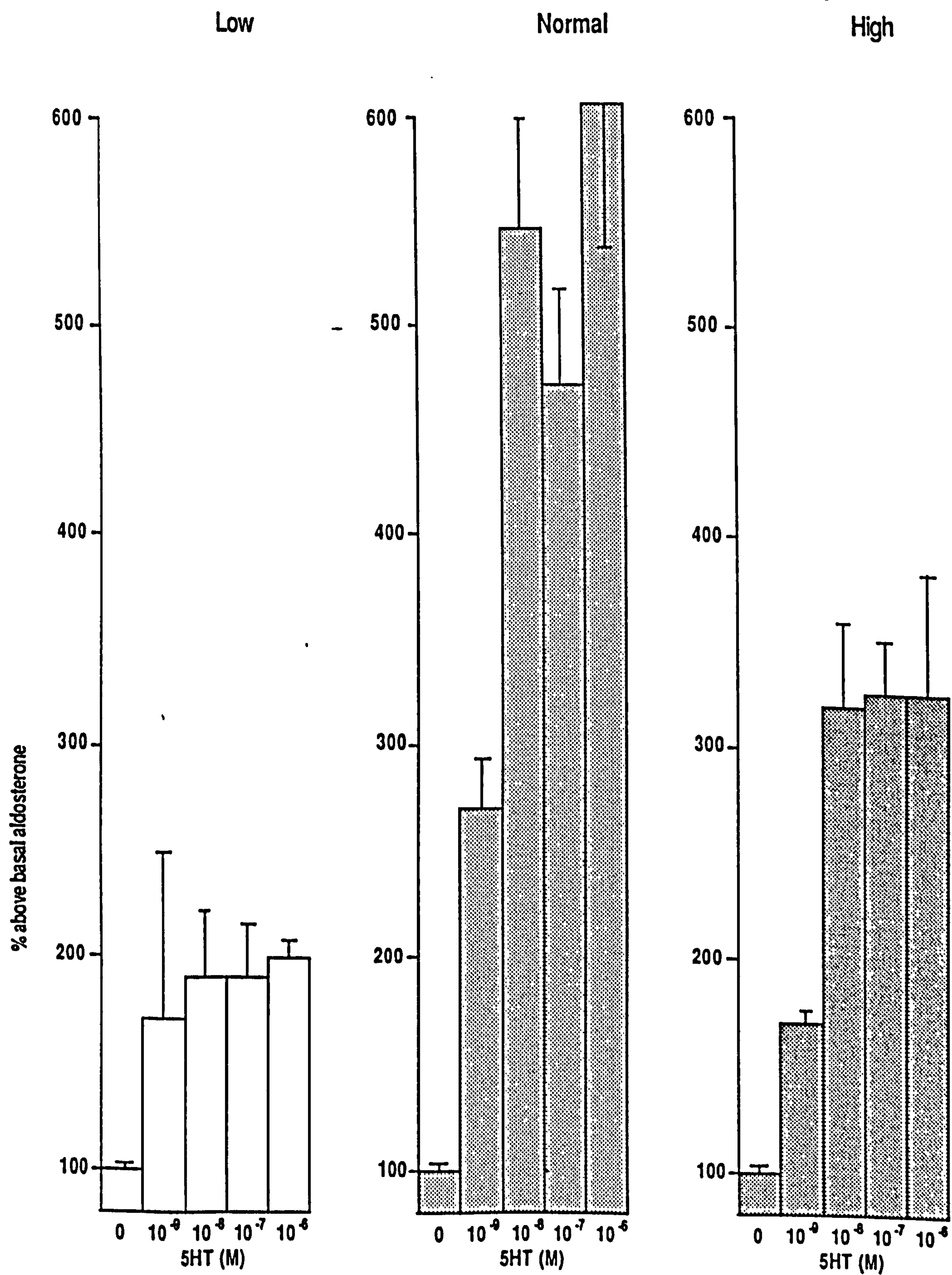


Fig 10.9c
% above basal aldosterone for All stimulation : 2 week salt diet
1st Experiment

High

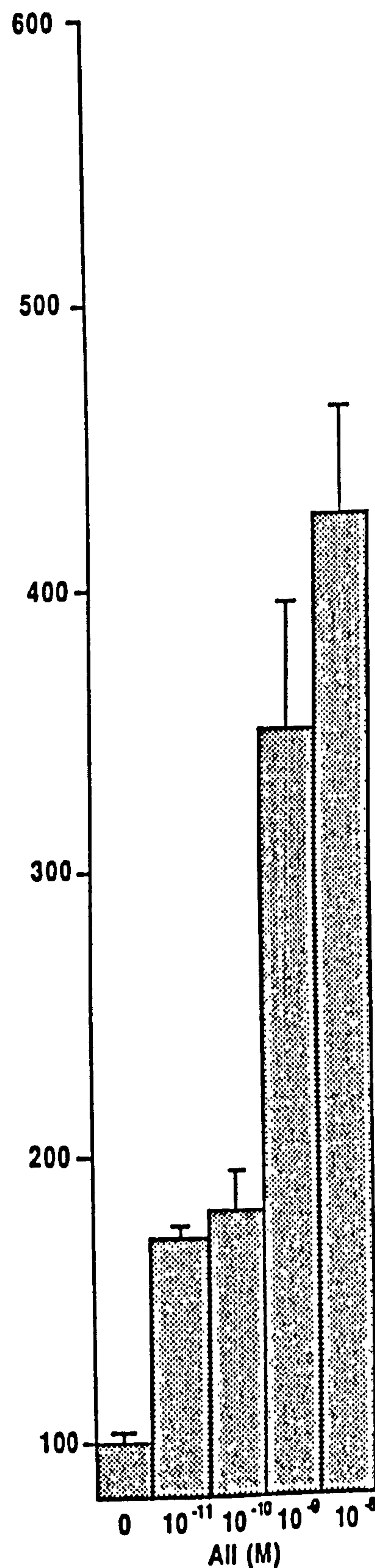


Table 10.16
p-values for % above basal aldosterone
: 2 weeks salt diet (1st experiment)

Stimulus (M)

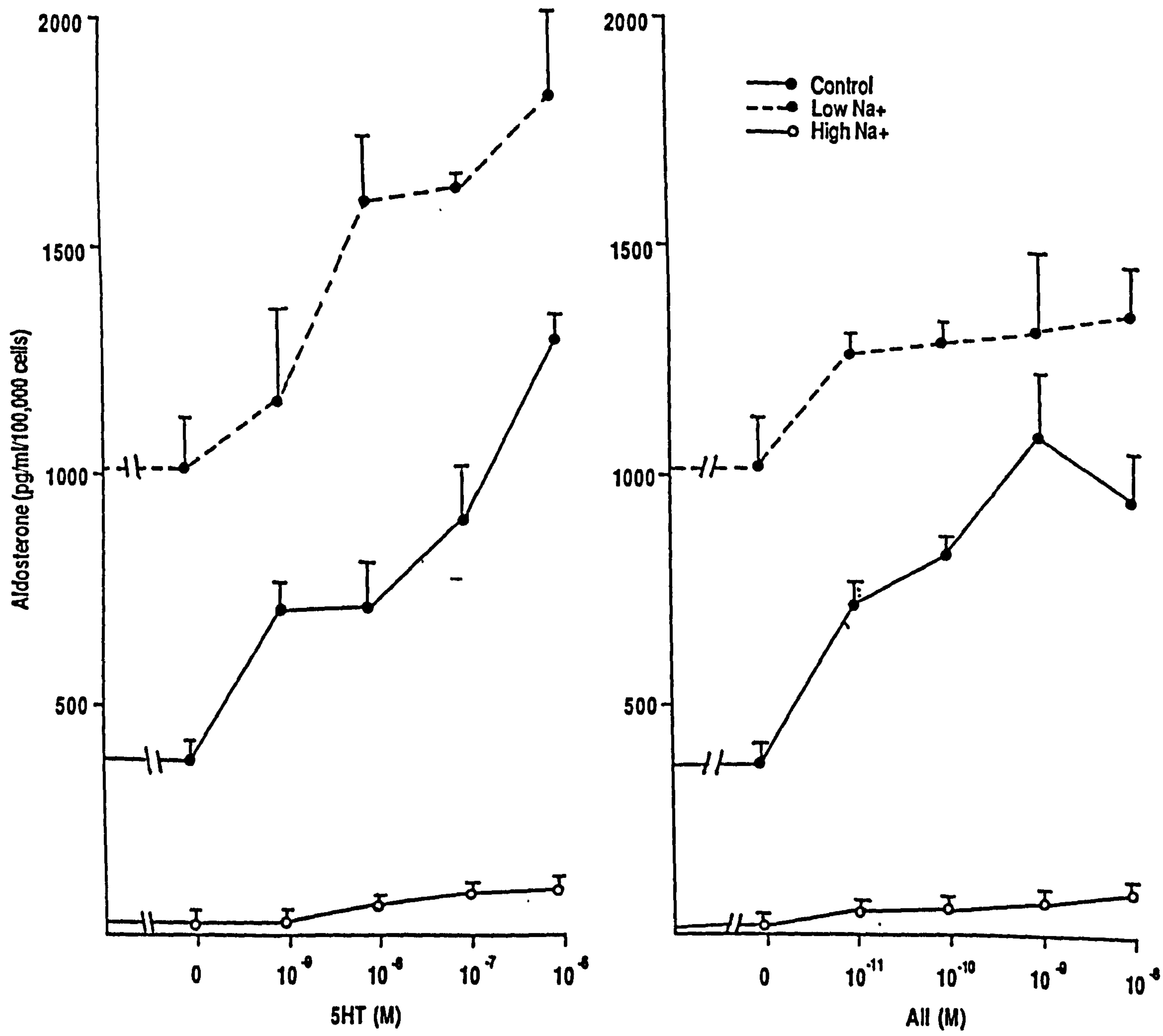
5HT

10 ⁻⁹	Normal/Low Normal/High Low/High	0.08 0.08 NS
10 ⁻⁸	Normal/Low Normal/High Low/High	0.005 0.02 0.07
10 ⁻⁷	- Normal/Low Normal/High Low/High	0.003 0.015 0.02
10 ⁻⁶	Normal/Low Normal/High Low/High	0.03 0.03 NS

All

10 ⁻¹¹	Normal/Low Normal/High Low/High	0.05 NS NS
10 ⁻¹⁰	Normal/Low Normal/High Low/High	NS NS NS
10 ⁻⁹	Normal/Low Normal/High Low/High	0.008 NS NS
10 ⁻⁸	Normal/Low Normal/High Low/High	0.005 NS 0.01

Fig 10.10a
Aldosterone dose response curves to 5HT and All : 2 week salt diet
2nd Experiment



control curve is not significantly different.

Figure 10.10a shows the second 2 week experiment with dramatic changes in basal steroid output according to salt status. Only in high salt does the sensitivity in terms of the absolute output response, decrease from 10^{-9} M 5HT in control and low salt, to 10^{-8} M 5HT; the AII response curve is reduced at all doses in the high salt situation. The percentage 5HT dose response for high salt is significantly reduced compared to control ($p < 0.02$) at 10^{-9} M 5HT but not affected at any other dose except 10^{-6} M 5HT; as indicated in an earlier interpretation, this is possibly a result of the large standard error bar measure of basal steroid output; in contrast the greater precision in basal steroid measurement in high salt diet, gives more accurate and interpretable results where there is a significant reduction in the sensitivity and percentage above basal steroid output at 10^{-11} M AII in high salt compared to normal salt status; there is no significant difference in the percentage above basal values at any other doses of stimuli, except at 10^{-8} M AII, in which case low salt diet has a lower percentage value (for the reasons above) than the control diet value at this dose.

10.3 DISCUSSION

Vinson et al., 1983 defined change in sensitivity as the lowest dose of stimuli required for stimulation and plotted steroid output as a percentage of maximal in studies on α -MSH stimulation. However the initial basis of these experiments was to give the same dose of stimulus to cell incubates in differing salt regimes and see how this

Fig 10.10b
% above basal aldosterone for 5HT stimulation : 2weeks salt diet
2nd Experiment

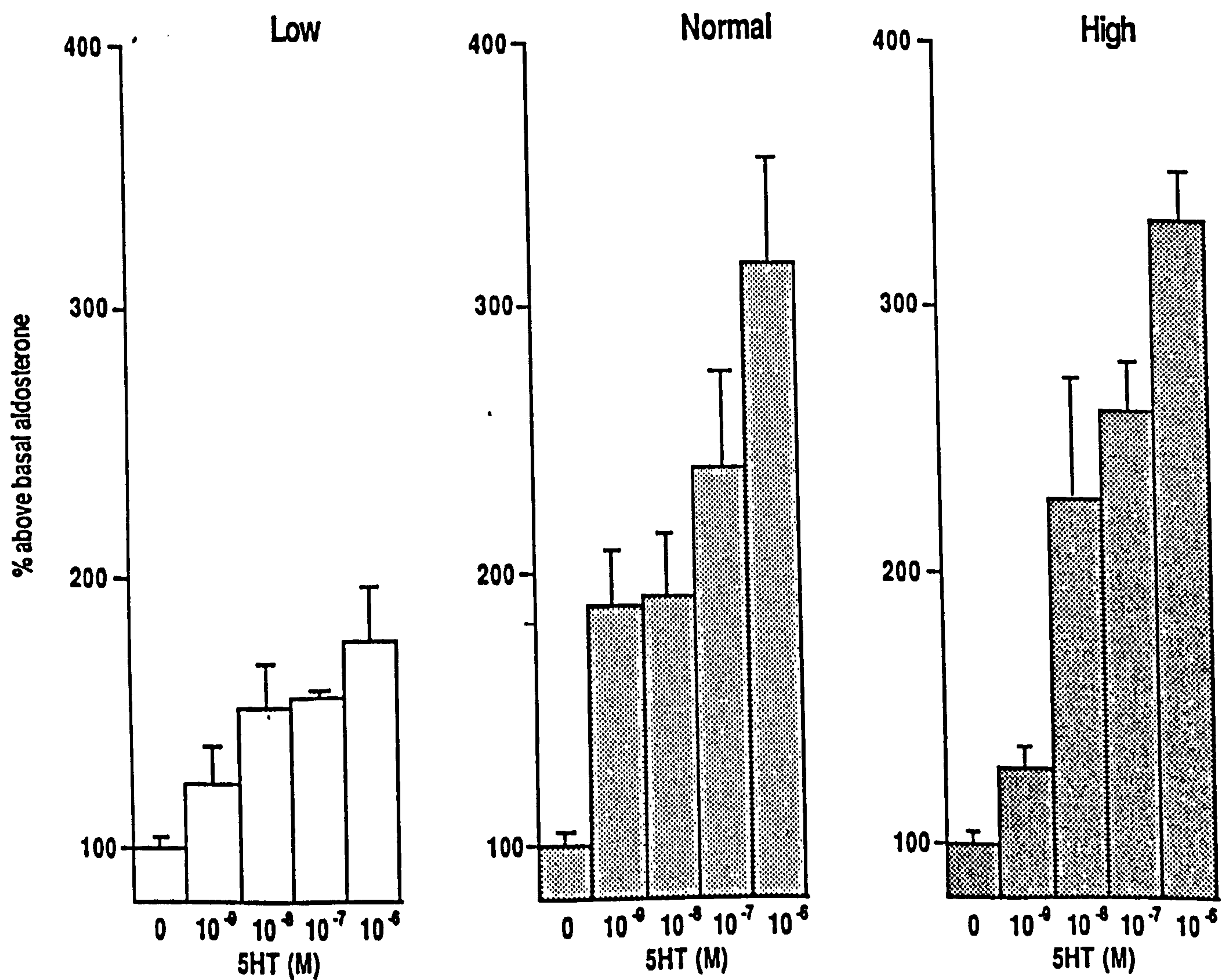


Fig 10.10c
% above basal aldosterone for All stimulation : 2 week salt diet
2nd Experiment

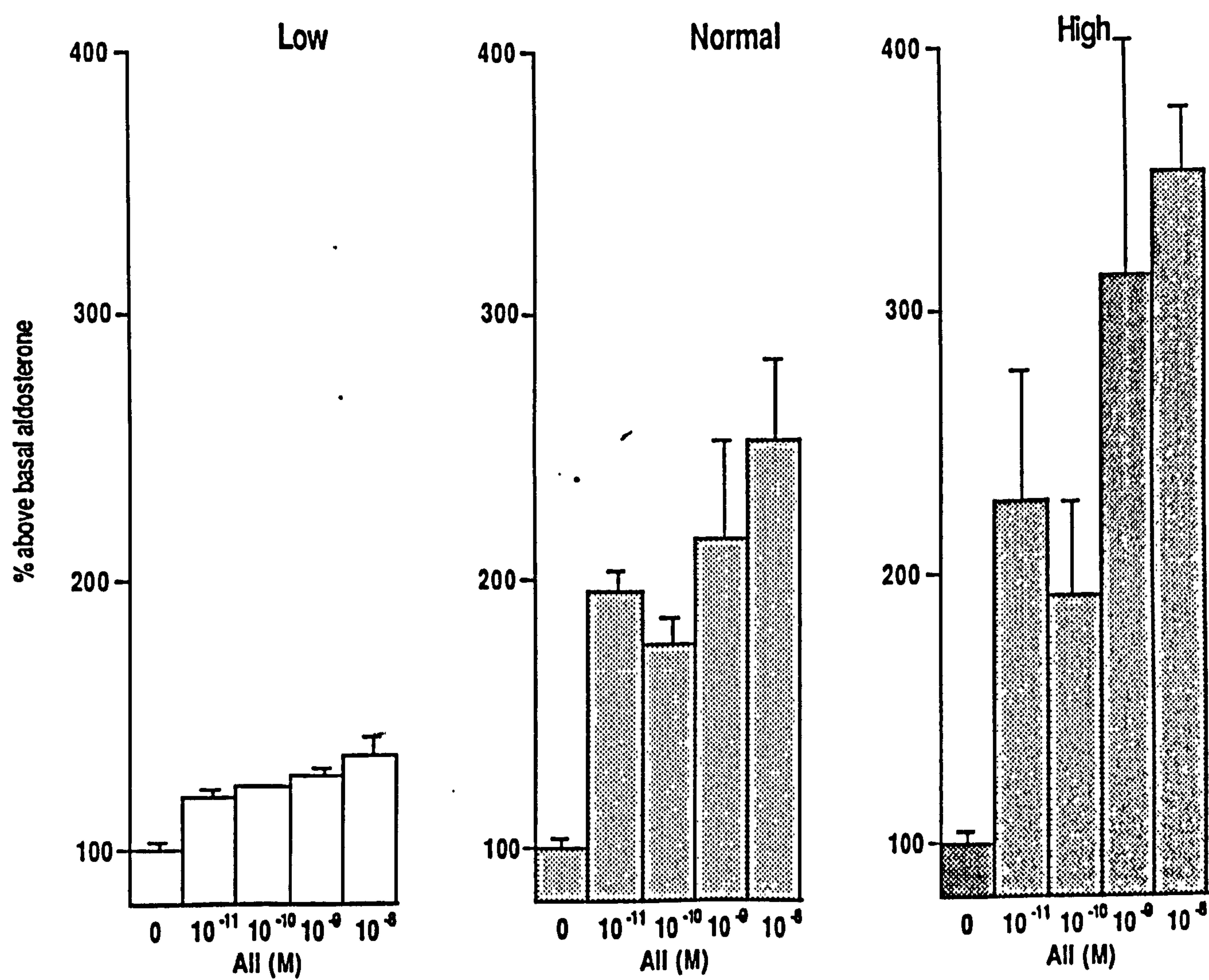


Table 10.17
p-values for % above basal aldosterone
: 2 weeks salt diet (2nd experiment)

Stimulus (M)

5HT

10 ⁻⁹	Normal/Low Normal/High Low/High	0.02 0.02 NS
10 ⁻⁸	Normal/Low Normal/High Low/High	NS NS NS
10 ⁻⁷	Normal/Low Normal/High Low/High	NS NS 0.02
10 ⁻⁶	Normal/Low Normal/High Low/High	0.03 NS 0.004

All

10 ⁻¹¹	Normal/Low Normal/High Low/High	0.0004 NS NS
10 ⁻¹⁰	Normal/Low Normal/High Low/High	NS NS NS
10 ⁻⁹	Normal/Low Normal/High Low/High	NS NS NS
10 ⁻⁸	Normal/Low Normal/High Low/High	0.01 NS NS

affected the magnitude of the response, both in absolute terms as Aguilera (Aguilera et al., 1978b) had set up and in percentage above basal terms.

In analysing both the in vivo and in vitro results, it can be seen that at 36 hours AII levels were significantly altered in response to salt status together with parallel changes in plasma aldosterone levels. At this time whole blood 5HT and adrenal 5HT levels were not significantly altered.

The basal plasma aldosterone level changes are also reflected in the in vitro experiments beginning at 36 hours. In both experiments at 36 hours, basal steroid output altered significantly with respect to control output; this was in contrast to Aguilera's findings (Aguilera et al., 1978b) who showed that for the same number of cells per incubate the basal aldosterone outputs were not altered by salt diet. In these studies, superimposing the absolute output dose response curves (in particular in the first 36 hour experiment), showed that the 'sensitivity' as defined by Aguilera as a change in the magnitude of the response and ED_{50} was increased in low salt compared to control dose response both for AII and 5HT; the converse was true for high salt relative to control. However, in the second experiment the sensitivity was not markedly changed although absolute basal values were again different with salt diet. Interestingly, these values plotted as percentage changes showed that overall, the percentage output were not significantly altered by salt status. However as indicated in the results section, this percentage measure

relative to a similar dose of stimuli on another salt diet regime, on reflection, is probably not a true measure of changes in responsiveness, since it measures values relative to the basal variability (which may not be determined with the same precision in the different salt states) within a given salt diet group, and the levels of significance are measured relative to the same dose of stimulus in another salt state, rather than relative to the basal or previous dose in the same salt regime. This anomaly becomes more apparent with greater steroid variability with the 2 weeks salt experiments although Muller (Muller et al., 1970) also observed these anomalies in his 1 and 2 week low salt diet experiments studying rat adrenal cortical responses in vitro in a static incubation system after a 2 week low salt diet regime. These percentage results may not be a direct measure of altered sensitivity on salt regimes but possibly an indirect consequence of steroid output variability and the adrenal steroid biosynthetic capacity. High basal steroid levels may 'exhaust' steroid precursor availability in increasing further steroid output in the zona glomerulosa with addition of stimuli, such as 5HT and AII described here, and thus give 'false' percentage values, and thus measures of response values.

Aguilera had suggested that this initial change in the sensitivity of the AII response was a result of changing AII receptor affinity, since this parameter was also measured at this time in her experiments (Aguilera et al., 1978b). In this case one could extrapolate that 5HT receptors are also present and subject to the change in sensitivity observed. However, without further studies

and binding analysis, this cannot be the whole conclusion. It is more than likely that the salt regimes have an all-encompassing effect on the activity of the enzymes involved in the steroid biosynthetic pathway, including 18-hydroxylase (Aguilera et al., 1980).

Further analysis at 4 days revealed a change in the responsiveness of both the AII and 5HT dose response curves, with no apparent shift in the curves, and thus sensitivity. Basal steroid levels again were markedly altered and these results appear to reflect Aguilera's findings for AII dose response curves alone. The anomaly arises in measurement of PRA and AII values which do not significantly change with salt status, although plasma aldosterone levels increase on low salt and decrease with high salt status. Interestingly, whole blood 5HT levels show a broad tendency to increase on low salt and decrease on high salt with respect to control diets, and although this is not statistically significant, the adrenal 5HT levels also show this trend being significantly different in the high vs. control group ($p < 0.05$). One could argue that 5HT at this time interval may play some subsidiary potentiating role in the control of plasma aldosterone levels in low salt diet in increasing the responsiveness of the adrenal to AII and 5HT in these limited studies. At this juncture it seems pertinent to mention the findings from Chapter 8, in which it was observed that changing endogenous 5HT levels in vivo affected the responsiveness of adrenal zona glomerulosa cells, both basally and stimulated levels. However if 5HT has any role to play at this time interval - and only more experiments would clarify this

- then its role is very slight at the local and humoral level. For it is also seen that after 1 and 2 weeks salt diet changes, plasma aldosterone, AII and PRA levels match the salt diet regime whereas both whole blood and adrenal 5HT levels are not significantly altered by salt status. Here again, the responsiveness as seen by superimposing the dose response curves, is the only parameter that changes, whereas the sensitivity is not altered for either 5HT or AII. The percentage above basal interpretation is not helpful in this analysis except perhaps in the high salt state. Sensitivity is an obscure parameter to measure but basal steroid level changes correlate overall with changes in AII plasma levels in these experiments, rather than any significant changes in 5HT levels. Aguilera (1978b) suggested that in rats, AII was the primary regulator of the adrenal responsiveness during altered Na^+ status since studies using rats fed low salt diets and captopril, to prevent AII formation, did not show an increase in plasma aldosterone; hypophysectomised rats also appeared to respond to a low Na^+ diet with an increase in plasma aldosterone although this was not as great as that seen in intact rats. The fact that AII levels changed after 36 hours would tend to suggest that AII is essential at the onset of altered Na^+ status, in the homeostatic control of aldosterone levels and that 5HT does not augment this in vivo. It is unlikely that acute Na^+ restriction would result in changes in the extracellular concentration of ions and in vitro changes in K^+ and Na^+ concentrations in the incubation medium within the physiological range do not appear to change the binding characteristics of AII to

zona glomerulosa cells (Douglas et al., 1976b., Fredlund et al., 1977). Circulating AII may influence AII receptor numbers or affinity even after such a short time but this may not necessarily correlate with changes in physiological responsiveness; for example Douglas et al., 1976a observed that within 19 hours of rat nephrectomy, adrenal AII receptors were reduced in number whereas the responsiveness was increased and the converse was found in uterine tissue; in a longer time study of 4 days altered Na⁺ status, it was observed in the monkey, adrenal AII receptors decreased, whereas there was an increase in adrenal sensitivity on low salt diets, so the time scale in the rat for changing sensitivity and/or receptor number, may not always change in the same direction. Indeed basal steroid levels were altered in these experiments after 36 hours compared to no change in Aguilera's study and she argued that AII receptor numbers did not alter after this time, hence similar basal levels; in Aguilera's study the normal rat diet contained 0.31-0.37% Na⁺, low salt diet contained 0.06-0.1% Na⁺, and high salt 2.8-3.1% Na⁺ and was investigated in a different strain of rat, (male Sprague-Dawley as opposed to female Wistar in these studies); the rats in these experiments were fed a wholemeal flour diet with 1% calcium carbonate in normal and low regimes (Fattah et al., 1977) which may have affected the basal steroid levels. That the basal levels of aldosterone are altered after 36 hours when AII levels are increased in low Na⁺ and decreased in high Na⁺, could be due to several factors as well as AII. Unless 5HT interacts specifically with AII receptors, the overriding influence on basal and stimulated

steroid response curves in vitro appears to be determined both by AII and Na^+ per se. Indeed in Man, Dawson-Hughes et al., 1981 showed that the dose response curves to infusions of AII in low salt diets was more sensitive than the dose response curve in AII-infused individuals. Other cases can be cited which show the same results, where low Na^+ affects the sensitivity of the zona glomerulosa to stimuli including the more physiologically important AII, K^+ and ACTH both in vivo and in vitro (Ganong et al., 1965; Kinson et al., 1968; McCaa et al., 1974; Palmore 1969; Pratt et al., 1981;). Further, one can argue that in the in vivo situation, the AII levels as measured in these experiments, change with salt status such that the adrenal is confronted with higher levels in low salt and lower levels in high salt, thus sensitivity as defined in in vitro experiments as a change in the effective lowest stimulatory dose cannot be equated with the in vivo doses, since they change accordingly anyway; thus Na^+ per se or another factor controls AII levels, which are therefore a joint or secondary controller of adrenal sensitivity (compared to Aguilera's description - see above) whereas Na^+ does not influence 5HT levels but the overall responsiveness to 5HT is controlled by another overriding factor i.e. AII and/or Na^+ .

In all species studied, low Na^+ causes an increase in pregnenolone biosynthesis and 18-hydroxylase activity, which is important in the final steps for conversion of corticosterone to aldosterone. In vitro studies carried out by Marusic and Mulrow (1975), demonstrated that short term Na^+ restriction caused an increased conversion to aldosterone in isolated mitochondrial studies. Similarly, Haning et

al., showed in 1970 that [^3H]-corticosterone was converted to [^3H]-aldosterone more markedly on low Na^+ diets compared to control. Fattah (1976,1977) also reported an increase in the conversion of 18-OHDOC and 18-OHB to aldosterone, in low salt status, and as originally suggested by Vinson et al., 1973, this could represent enhanced binding of the non-diffusible steroid (18-OHDOC), to an endogenous protein in the adrenal that allows greater enzymic conversion to aldosterone through 11- β -hydroxylation. In vivo, the direct stimulatory effects of Na^+ on adrenal responsiveness was demonstrated in the dog, an acute lowering of plasma Na^+ by infusion of 5% glucose into the arterial supply of the isolated adrenal of hypophysectomised-nephrectomised dogs (Davis et al., 1963) or into the arterial supply of adrenal autotransplants in the neck of sheep (Blair-West et al., 1962) caused a prompt rise in aldosterone biosynthesis. Davis et al., 1968 and Tait et al., 1970 have suggested that Na^+ ions stimulate aldosterone biosynthesis at a step lying between corticosterone and aldosterone as well as between cholesterol and pregnenolone. Aguilera also demonstrated that AII infusions over 4 days, could almost reproduce the effects of low Na^+ diet on the activity of 18-hydroxylase, plasma aldosterone and AII receptor numbers (Aguilera 1980). AII is also known to have a trophic effect on the zona glomerulosa which augments an increase in mass stimulated aldosterone biosynthesis, but the effects are linked to a chronic influence and could include the 36 hours study. In the converse situation in which aldosterone levels are suppressed by changing salt diet after 36 hours, AII levels are suppressed, but

Aguilera (1984) had previously shown that infusions of AII in high salt status, could not increase adrenal responsiveness or plasma aldosterone levels to control values. Thus it has been argued that other modulators independent of reduced AII levels, in high salt status, are responsible for the changing sensitivity and aldosterone biosynthetic capacity of the adrenal gland. In infusion studies with AII or metoclopramide (dopamine antagonist) infused separately into rats for 48 hours on high salt diets, there was no change in plasma aldosterone, 18-hydroxylase activity or AII receptor number but coinfusion of MCP and AII, elevated plasma aldosterone levels to levels observed after AII infusions into control rats (Aguilera et al., 1984); a comparable observation has also been observed in vivo in Man (Gordon et al., 1983). In measuring 5HT levels on high salt, which could also possibly influence the adrenal responsiveness in high salt status, 5HT levels did not appear to alter after any time interval. Further experiments in which 5HT concentration per number of platelets were made, would probably give a better index as to changes in levels with salt status. Having said this it has been cited in Nahmod's paper (Nahmod et al., 1978) that Goldstein (1973) found that AII stimulates the release of 5HT from mast cells, further Osim et al., 1983 was able to show that prelabelled platelets carrying [14 C]-5HT differentially deposited [14 C]-5HT into gut, thyroid and the adrenal gland, and this was enhanced after 5HTP treatment with resultant increase in 5HT biosynthesis. In this regard AII in the brain was also found to increase tryptophan hydroxylase activity with a resultant increase in

5HT biosynthesis (Nahmod et al., 1978). The possibility exists therefore, that at 4 days a critical point in 5HT turnover - in fact 3-4 days is the half-life of a platelet- and release in adrenal tissue or associated mast cells and platelets is reached, which is manifest as an increase in 5HT concentration with changes in extracellular volume. At longer time studies, AII and or Na^+ may increase the biosynthesis of 5HT in brain and peripheral regions including the adrenal gland and/or increase the release of 5HT from mast cells within the adrenal connective tissue. However the overall turnover and metabolism may balance out, since as indicated in the metabolism chapter, the adrenal gland appears to have a high monoamine oxidase activity. In a dynamic study, the possibility that release of 5HT is increased at the adrenal over a shorter time scale in low salt status with the converse occurring in high salt status, may describe and contribute to some of the changes seen in the adrenal basal and stimulated levels of steroid output; this hypothesis can only be adequately tested by studying rats kept on altered salt regimes with altered endogenous 5HT levels imposed such as used in Chapter 8.

That whole blood 5HT at the level of the adrenal and measured in platelets, did not appear to altered significantly at any time except after 4 days, may indicate that 5HT has indirect modulatory effects at higher control centres, or that 5HT levels on a platelet count basis may need to be investigated to show any change. In the case of primary hyperaldosteronism, Gross et al., 1981 showed that the increased adrenal sensitivity to AII could be reduced by

administration of the 5HT antagonist, cyproheptadine, and suggested that central serotonergic mechanisms either directly or by stimulating the release of other central factors, could augment an increased adrenal sensitisation state in hyperaldosteronism that paralleled that seen in low Na^+ status. Several groups have shown that other mechanisms exist to regulate aldosterone secretion as well as AII. In the rat, Palmore (1969) demonstrated that rats kept on a low Na^+ diet for 8 days, secreted aldosterone even after 18 hours post-bilateral nephrectomy; further there was a 100% increase in aldosterone secretion following Na^+ depletion by peritoneal dialysis in Na^+ replete-nephrectomised rats, which suggested a non-renal factor in the control of plasma aldosterone.

Adrenal renin has also been postulated to be important in modulating adrenal sensitivity in low salt status. Doi et al., 1984 was able to show a positive correlation between adrenal renin concentration and plasma aldosterone in nephrectomised rats and thus independent of renal renin, but this does not alone argue a formidable regulatory role in aldosterone biosynthesis on altered sodium regimes.

In nephrectomised dogs on low Na^+ diets, McCaa (McCaaet al., 1974) observed that the plasma aldosterone response to infused AII could be maintained provided the pituitary was intact; removal in nephrectomised-hypophysectomised dogs prevented an increase in plasma aldosterone in low salt status which could be restored by administration of pituitary extracts. As mentioned previously, pituitary factors may be involved in controlling plasma aldosterone levels on altered Na^+ diets. McCaa et al., 1974 showed that

nephrectomised humans retained the capacity to maintain basal aldosterone levels in the absence of the renin-angiotensin system and could increase plasma aldosterone levels in response to all physiological stimuli. The pituitary factor(s) were not ACTH or growth hormone or its releasing factor, since growth hormone deficient patients are able to respond to increasing plasma aldosterone levels on low salt diets, whereas in panhypopituitary patients administered somatotropin, the aldosterone response was not restored and this suggested other pituitary factor involvement. Coghlan et al., 1979 was able to show in Na⁺ depleted sheep, an increase in plasma aldosterone within minutes of drinking a bicarbonate solution and suggested a central neural involvement in altering aldosterone levels in salt depletion.

Idiopathic hyperaldosteronism and the increased plasma aldosterone response curve to physiological stimuli including ACTH and AII has been postulated as caused by overactive serotonergic pathways stimulating pituitary factor release (Gross et al., 1981, Shenker 1985). In low salt status, AII levels are increased both peripherally and in the brain and in the latter region are known to increase 5HT biosynthesis within serotonergic neurones so this would be in keeping with an increased central serotonergic involvement. The converse may hold true for high salt diets, or may be related to an increase in other humoral factors such as ANF, which inhibits the AII response in normal salt status adrenals in vitro (Chartier 1984), and is released from atrial granules during volume expansion and/or high salt states (Atarashi 1984); or dopamine both peripherally

and/or centrally. In this regard Alexander (Alexander et al., 1974) noted an increase in dopamine excretion in high salt status parallel to an increased synthesis of the amine in high salt. Dopamine at the level of the adrenal may exert the tonic inhibitory influence independent of 5HT levels, which appear not to change, or within the central regions, increased dopamine production may exert an inhibitory effect on 5HT release from serotonergic terminals impinging on pituitary cells.

That 5HT levels do not appear to change with salt diet, does not rule out a dynamic, local paracrine role of 5HT in modulating adrenal sensitivity, since measurements of 5HT in these studies only gives an overall static picture of levels. It could be argued that 5HT biosynthesis may be increased on low Na^+ diets with AII levels increasing biosynthesis, since tryptophan hydroxylase is present (albeit in low amounts) within adrenal tissue (see Chapter 8); additionally, contaminating mast cells may release 5HT more readily after Na^+ depletion, and would thus modulate adrenal responsiveness. Equally however, mast cells could take up 5HT more avidly in low salt diets although investigations are needed to confirm this theory, so the average concentrations within the adrenal gland would appear not to change. In the high salt state, 5HT synthesis may be depressed by lower circulating AII levels, but if dopamine exerts a local inhibitory influence on 5HT release the overall picture would be of no change in 5HT levels within the adrenal. Thus, dynamic changes are possibly the key to determining a local paracrine role of 5HT within the adrenal gland in altered Na^+ status. The effects of salt

status on platelet uptake of 5HT may also be an important local factor in determining adrenal responsiveness although there does not appear to be reports of such studies being done. That the effects of salt are rapid and dynamic, would tend to argue that if 5HT has a modulatory role, the turnover and synthesis would parallel these changes. One limiting factor would be the localisation and specific activity of tryptophan hydroxylase, the rate limiting step in 5HT biosynthesis. As mentioned previously (Chapter 9) the enzyme is present in very low amounts within the adrenal and is more abundant in central regions, thus it would be more reasonable to suggest a central rather than a direct local adrenal involvement of 5HT in the altered sensitivity of the adrenal.

Chapter 11

IMMUNOHISTOCHEMICAL STAINING FOR SEROTONIN IN THE ADRENAL

11.1 Immunohistochemical Staining - Binding of the 5HT Antibody

The immunochemical procedure as applied to biogenic amines such as 5HT, is based on the phenomenon of small molecules, which by themselves are completely devoid of antigenicity, becoming immunogenic after coupling to a carrier protein (Landsteiner 1947). With the advent of the raising of specific monoclonal antibodies to 5HT, it has become possible to localise 5HT containing cells in several species of animal tissues. The most extensive application of this has been in rat brain and the central nervous system (CNS) (Buffa et al., 1980., Steinsbusch et al., 1978) and also in enterochromaffin cells in the gut lining of rat (Nilsson, et al., 1985) and in pancreatic beta cells in the guinea pig (Lundqvist et al., 1978) to quote but a few examples. The 5HT antibody in most instances, is coupled to a paraformaldehyde molecule, since fixation of tissues is most optimal with this fixative. Paraformaldehyde forms methyl bridges with amino groups within peptide bonds in the tissues, but only with the -NH_2 groups i.e. at a high pH close to the pKa value for the amino acids $\text{R-NH}_3^+ = \text{R-NH}_2 + \text{H}^+$

The reaction is



Hence, the concentration of fixative and the pH, are critical factors in determining how effective the localisation of antigen in the tissue will be. The degree of fixation and the temperature, determines the intensity of staining due to localisation of the

antigen (Berod et al., 1981). Because of this fixation treatment, all tissue is made accessible for antibody attachment previously coupled to paraformaldehyde.

11.2 Detecting 5HT Binding

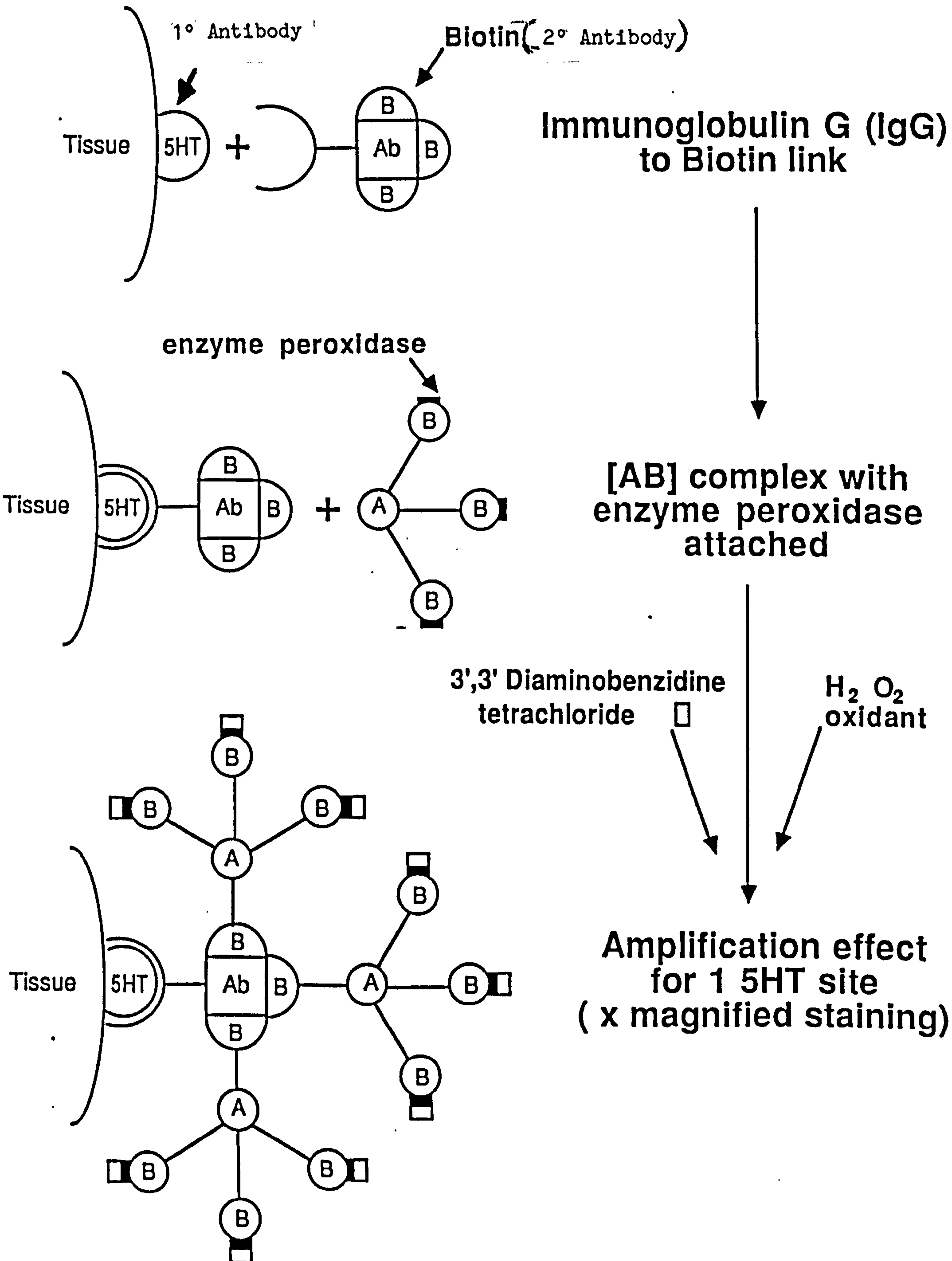
Once the antibody binds its specific antigen, it becomes necessary to detect the immunoreaction, and there are two ways in which this can be done: 1) direct staining in which the antibody is attached to a detectable marker; 2) indirect staining or the sandwich technique, in which the primary antibody i.e. that binding to the antigen, acts as an antigen to the secondary antibody linked to a detectable visual marker, normally an immunoglobulin. The markers can be heavy metals, fluorophores or enzymes. In this case horseradish peroxidase was used to activate the detection of the dye, 3',3'-diaminobenzidine tetrahydrochloride (DAB), attached to biotin molecules, in the very sensitive avidin-biotin (ABC) technique described below.

11.3 The Avidin-Biotin Technique

The avidin-biotin interaction in immunoenzymatic techniques provides a simple and sensitive method to localise the antigens in the formalin and paraformaldehyde fixed tissue preparations. The initial reaction requires (as in other primary techniques) the localisation of the antigen, and in this case 5HT, which is treated with a 5HT antibody. Biotin is a small vitamin molecule which is coupled to the antibody. This coupling of biotin to an antibody or an enzyme peroxidase (in the final stage), makes it capable of

Fig 11.1

Binding of Avidin - Biotin to 5HT



binding avidin molecules. Avidin is a 68, 000 kDa glycoprotein found in egg white, and with a very high affinity for biotin ($K_d 10^{-15}M$) as well as multiple binding sites for biotin.

The next stage can be modified with either sequential additions of avidin, followed by the biotin-peroxidase coupled molecule (indirect bridged avidin biotin technique (IBAB), or by complex addition of avidin-biotin peroxidase in pre-determined ratios - the ABC technique. The ABC technique is the method of choice in the reaction shown (Fig.11.1).

For immunohistochemistry, the tissue must be previously fixed. The choice of fixative in most cases is 4% paraformaldehyde and can either be perfused into the tissue by intracardiac perfusion or post-fixed by removing tissue slices and then post-fixing the tissue slices in fixative solution or vapour as used here, placing fresh tissue sections in a closed chamber containing paraformaldehyde solid.

11.4 METHODS

11.4.1 Tissue Preparation

Female Wistar rats (180-200g) were killed by cervical dislocation and whole adrenals removed and immediately transferred to a metal beaker containing isopentane (antifreeze), suspended in a flask of liquid nitrogen. Whole glands were kept at $-70^{\circ}C$ until processing for tissue sectioning.

11.4.2 Mounting of Tissue Sections

The adrenals were transferred to mounting blocks and fixed in position with a few drops of tissue fixing solution, Depex (mounting medium, Gurr Microscopy Materials, BDH) placed on the top of small cork blocks and left to set, before securing the adrenals using a pair of fine forceps, into position on top of the cork surface. This manoeuvre allowed anchorage of the tissue for cutting thin sections, once they had been loaded into the cryostat. All procedures were carried out at approximately -20°C using a water bath of dry ice and absolute alcohol. The cryostat model (HR Mark 2 Cryostat, Slee, London) was kept refrigerated at a -25°C and the mounting blocks with fresh tissue, placed at the correct angle for cutting with the cryostat blade. The blade cutting width was set at $8\ \mu$ (Hökfelt, et al., 1981., Verhofstadt et al., 1983) and sequential tissue sections collected from the cryostat blade by placing a pre-cooled chrome-alum coated glass slide over the blade surface to allow adsorption of tissue slices onto the slides. The slides were then gently lifted off and placed into slide trays containing paraformaldehyde powder. Tissue slides were not completely immersed, but rather equilibrated with paraformaldehyde vapour for 24 hours at 4°C , to allow post-fixation of the tissue slices. This was thought to be the best method of fixing such thin tissue sections. Previous experimentation using whole adrenals perfused with fixative prior to cutting, caused brittleness to the tissue, making it intolerant of thin sectioning treatment. Brain tissue from the

dorsal raphe (Osborne 1982) region was also post-fixed and cut at 25 μ as previously carried out in other studies (Steinsbusch et al., 1978); this acted as a control for 5HT staining since the technique using these reagents has already been optimised in this tissue.

11.4.3 Preparation of antiserum

Antibodies to 5HT-BSA were raised in rabbits (5HT rabbit antibody - Immunonuclear Corporation, Stillwater, Minnesota) as described previously (Steinbusch et al., 1978). The specificity has already been determined and studied in detail. The antiserum reacts with 5HT and 6-hydroxy-tetrahydro- β -carboline, which is the form of 5HT in formaldehyde fixed tissue.

11.4.4 Preparation of Secondary Antibody

Anti-rabbit immunoglobulin G (IgG) was raised in goat serum and coupled to biotin, as described above; the ABC kit was purchased from Vector U.K. (Vectastain ABC).

11.4.5 Immunocytochemical Procedures

The post-fixed tissue slides were placed in tissue slide trays and thoroughly rinsed with phosphate buffered saline (PBS) and then incubated at room temperature for 1hour in 20% goat serum. This was thought to be the optimal concentration of serum which would reduce the non-specific binding of 5HT antiserum in the tissue. The anti-rabbit antibody circulates in goat serum, which also contains several plasma proteins capable of binding to 'foreign' matter and treating it as antigenic. Hence, drowning out these potential antigenic sites in the tissue section with goat serum (untreated with anti-rabbit

antibody), allows correction and optimisation of binding to the serotonergic sites of interest. Sections were washed three times in PBS and then incubated with 5HT antiserum at a final concentration of 1:200 at room temperature for 48 hours. The slides were then rinsed in PBS with azide and Triton (0.075 %) - to ensure antibody penetration through the cell membrane - twice for 5 minutes each. The biotin - antibody moiety was then added at 1:200 concentration in PBS azide and Triton, for 45 minutes at room temperature. PBS without azide or Triton (since at this stage it affects the final stage reaction), was then used in two 5 minute rinses of the treated slides. At this stage, a series of sections were 'primed' with a peroxide solution (1 - 3% peroxide solution in 30% methanol diluted in PBS) in 100µl aliquots, to inactivate the red blood cell enzyme, catalase, which reacts with peroxides. Thus, this reduces the non-specific staining, since the DAB complex may cause a colorimetric reaction when red blood cells (containing peroxidase activity) react with it; therefore specific 5HT containing cells treated with the enzyme-linked biotin complex would be drowned out with colour staining of DAB to endogenous catalase-linked reactions within red blood cells. The sections treated in this way were incubated with hydrogen peroxide (1 - 3%) for 30 minutes after which time, the slides were given 3 x 5 minute washes in PBS and then treated in the same way as the 'non-primed' sections, with the AB complex. The AB complex was made up by addition of 1:100 final concentration of solution A in the Vector kit to 1:100 of B (avidin and biotin with the enzyme peroxidase attached), and this was introduced onto the

slides and incubated for 30 minutes at room temperature. The slides were then rinsed in 50 mM Tris buffer (pH7.4) twice for a further five minutes each before the colorimetric enzymatic reaction using 3',3-diaminobenzidine tetra chloride (DAB) took place. DAB (Polysciences) is sold in bottled solid form, and in the anhydrous state is carcinogenic. All steps in its final aqueous preparation were therefore carried out in a fume cupboard. A 10 ml solution containing Tris buffer, was injected into the top of the rubber-topped bottle of 5 mg DAB powder (0.5 mg/ml) and thoroughly mixed in the fume cupboard. A few drops of this solution were placed onto the treated slides in the fume cupboard and left to incubate at room temperature for 10 minutes. After this time, a further addition of this mixture containing 140 μ l of H₂O₂ (3%) - the reducing compound required for the colour reaction - was added to the preformed mixture and incubated on the slides for a further 10 minutes. Two 5 minute washes followed in Tris buffer and the treated slides were mounted and dried for 6 - 8 hours. Slides were then dipped into increasing concentrations of absolute alcohol starting at 50% alcohol concentration up to two immersions in 100% alcohol. Each immersion was for approximately 2 minutes. This procedure removed both extra- and intracellular water from the tissue. The slides were then placed in a solution of the non-aqueous medium, xylene, before finally being mounted. Slides were placed in slide holders at a slight tilt and a few drops of Depex were placed on to the tissue. The cover slip was then gently lowered over the solution, to allow diffusion uniformly and without air bubbles, across the whole

of the cover slip. The slides were then left to set for at least 4 hours before being visualised under the light microscope.

Several sections of post-fixed adrenal tissue were treated with only haematoxylin and oil red O stains for the differentiation of cell structure and lipid deposition respectively.

11.5 Haematoxylin Staining for Cell Structure

11.5.1 Materials

Haematoxylin 1 g was dissolved in 10 ml of absolute alcohol and 20 g of potassium alum previously dissolved in hot water was added. The solution was boiled, and 0.5 g of mercuric acid added, to 'ripen' the stain, until the solution turned dark purple. The solution was cooled rapidly under tap water and filtered into stock bottles and left for several hours.

Eosin stain was prepared by mixing 1g Eosin powder to 100 ml of distilled water.

11.5.2 Staining Method

Tissues slides were placed in a solution of 5% acetic acid/ethanol and then removed into distilled water after a couple of minutes. After washing, the slides were placed for 2 minutes in the haematoxylin stain. The deep purple stain on the slides became a deep blue when they were later dipped into hot water (60°C). The slides were then put into cold distilled water; immediately afterwards, the slides were quickly dipped in and out of Eosin stain and washed twice in cold water.

The dehydration process involved sequential dipping into alcohols of increasing concentration (50%, 90%, and 2 x 100%). The slides were then immersed into xylene and mounted.

11.6 OIL RED O METHOD FOR COLOURING FATS

11.6.1 Stock Solution of Oil Red O

A saturated solution (0.5%) of oil red O in absolute isopropyl alcohol is required. Oil red O is a strong colourant of fats, but it requires a high concentration of fatty matter to be drawn out of its alcoholic solution in the section, and is appropriate to use in studying adrenal steroid-producing cells.

11.6.2 Staining Solution

A stock solution of oil red O (60ml) was mixed with distilled water (40 ml) and kept indefinitely after filtering .

11.6.3 Staining Method

Fresh sections must be used in this staining procedure. Sections were rinsed in 60% isopropyl alcohol. They were then immersed in oil red O solution for 10 minutes and briefly rinsed in 60% isopropyl alcohol.

At this stage, the sections were washed in distilled water and then counter-stained in haematoxylin stain for a further 1 minute, before being dried and mounted.

A few drops of warmed glycerol solution was placed on top of the tissue slides, and the coverslip slowly put into position before dehydration treatment as outlined above.

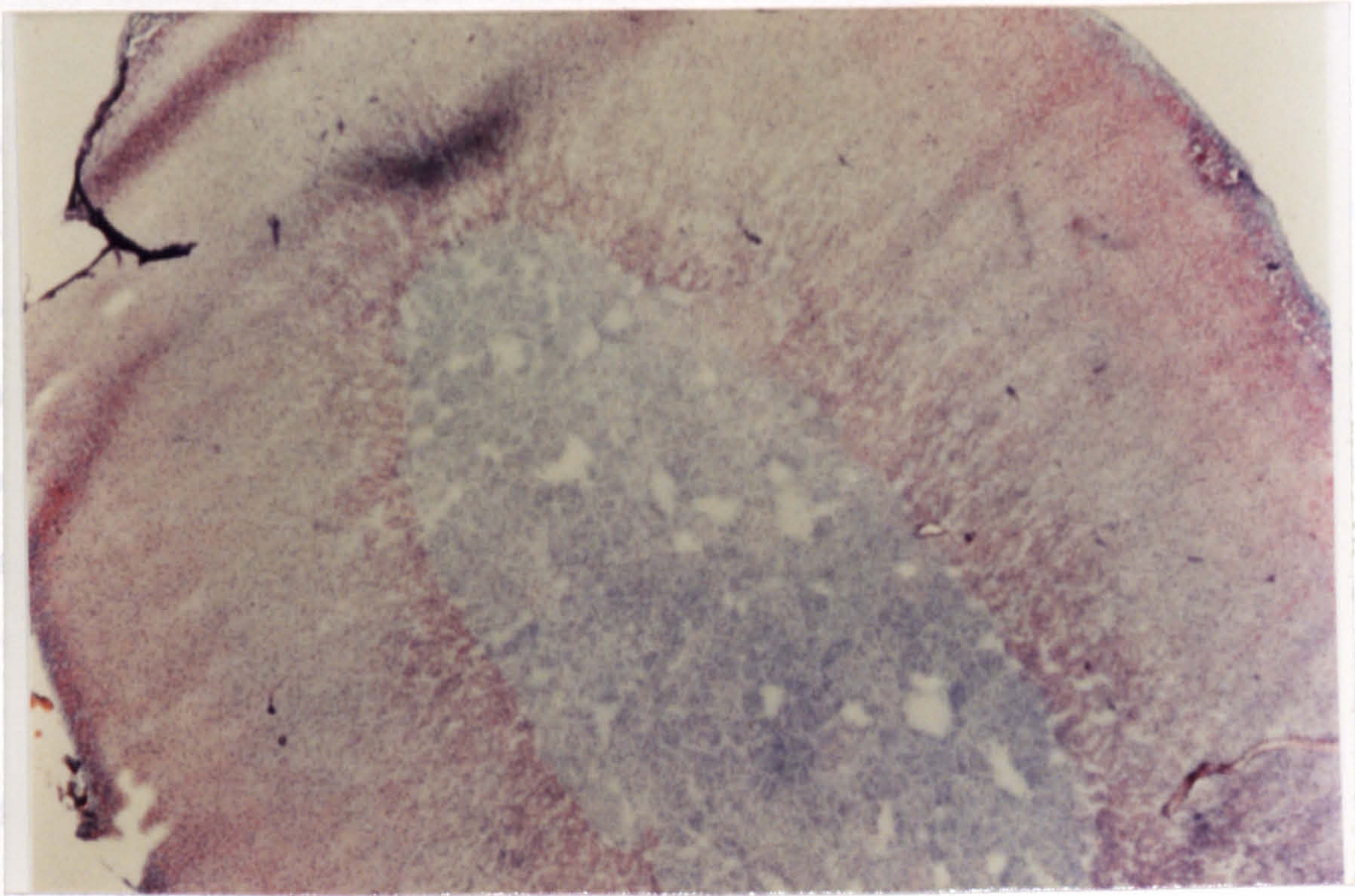


Figure 11.1

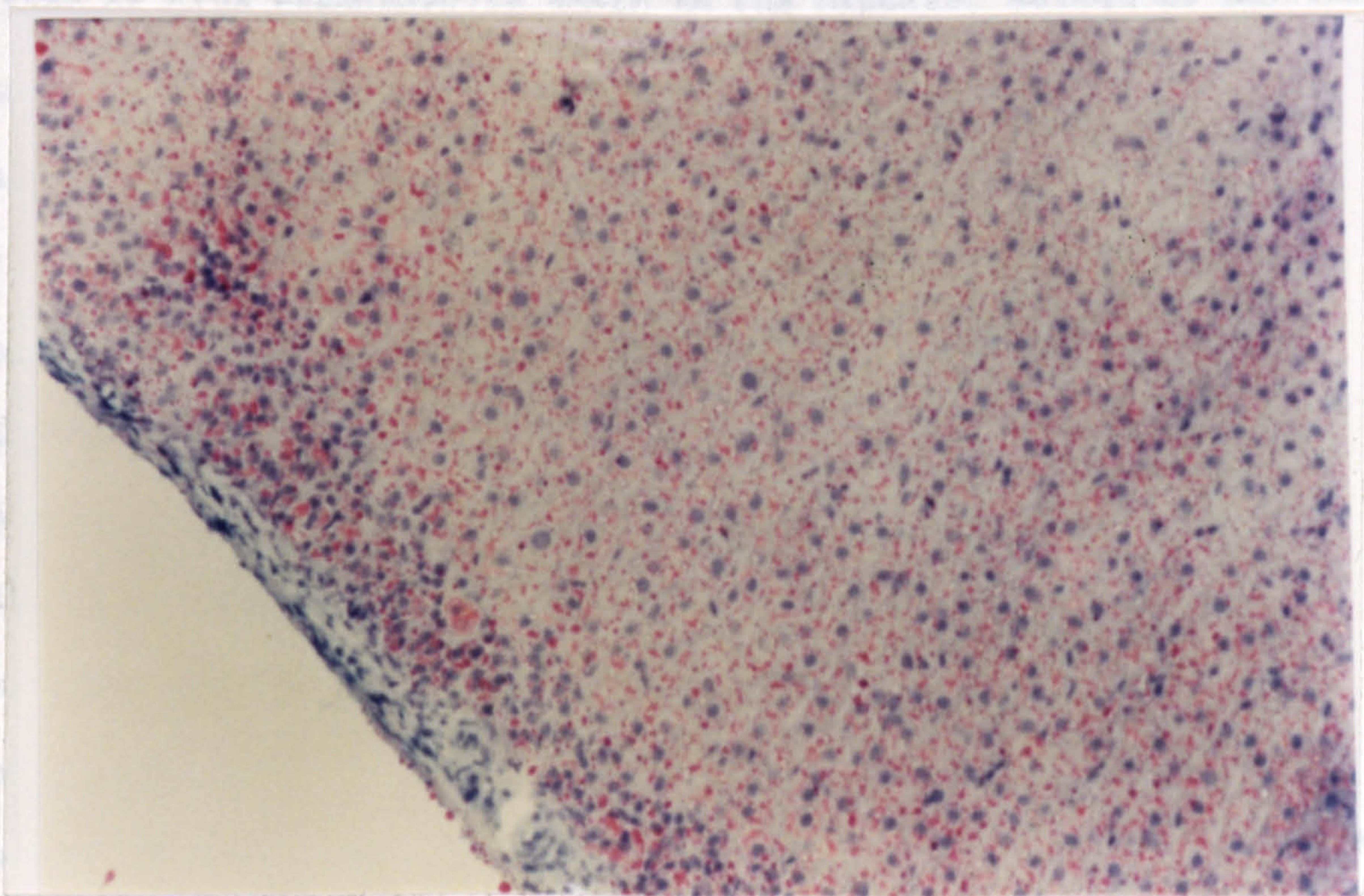


Figure 11.2

11.7 LIGHT MICROSCOPY

Sections were examined and photographed using a Nikon biological microscope (Optiphot), at the magnifications quoted (see Results) and corrected up for printing and developing.

The equation for the magnification process is:

Magnification of objective (variable) x Magnification of projection lens (constant 2.5) x 16 where 16 = the magnification of the transparency up to print size.

11.8 RESULTS

11.8.1 Haematoxylin and Oil Red Staining

Figure 11.1 shows a low powered section (x 160) of the gross morphology of the rat adrenal gland in transverse section. There is a band of blue haematoxylin stain in the capsule, devoid of any oil red O staining, since steroid is not present in this region. The whole of the cortex, from the zona glomerulosa zone to the zona reticularis, is stained with both haematoxylin and oil red, demonstrating that steroid is present in all cortical zones. However, there is no clear cut differentiation between the amount of steroid present in varying regions, although it is known that the largest amounts of steroid are present in the zona fasciculata, the intermediary zone concerned principally with corticosterone production in the rat. The central blue stained region corresponds to the steroid free medullary zone. In all regions, there are varying sizes of cortical and medullary sinusoids, demonstrating the multiple networks of the

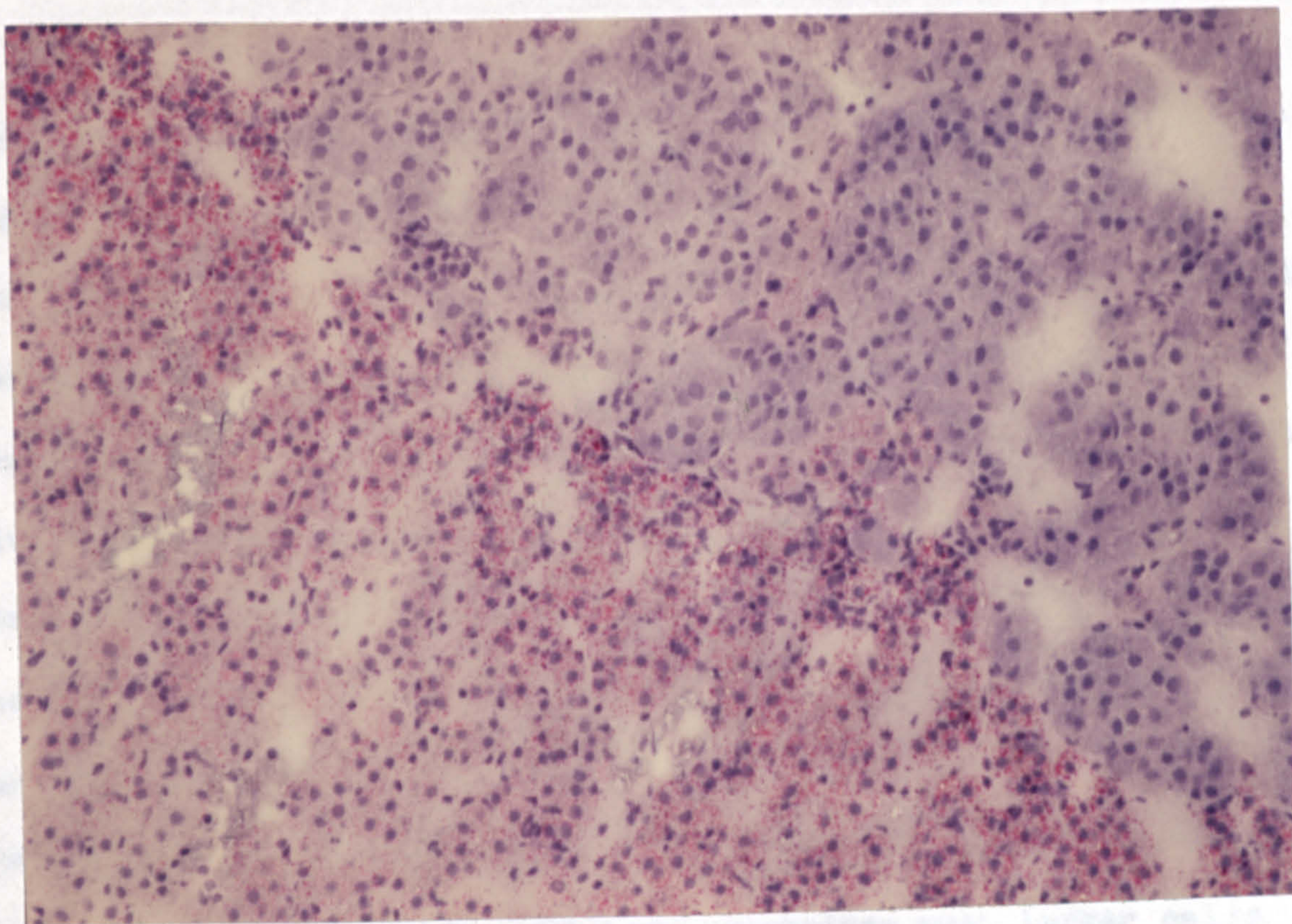


Figure 11.3

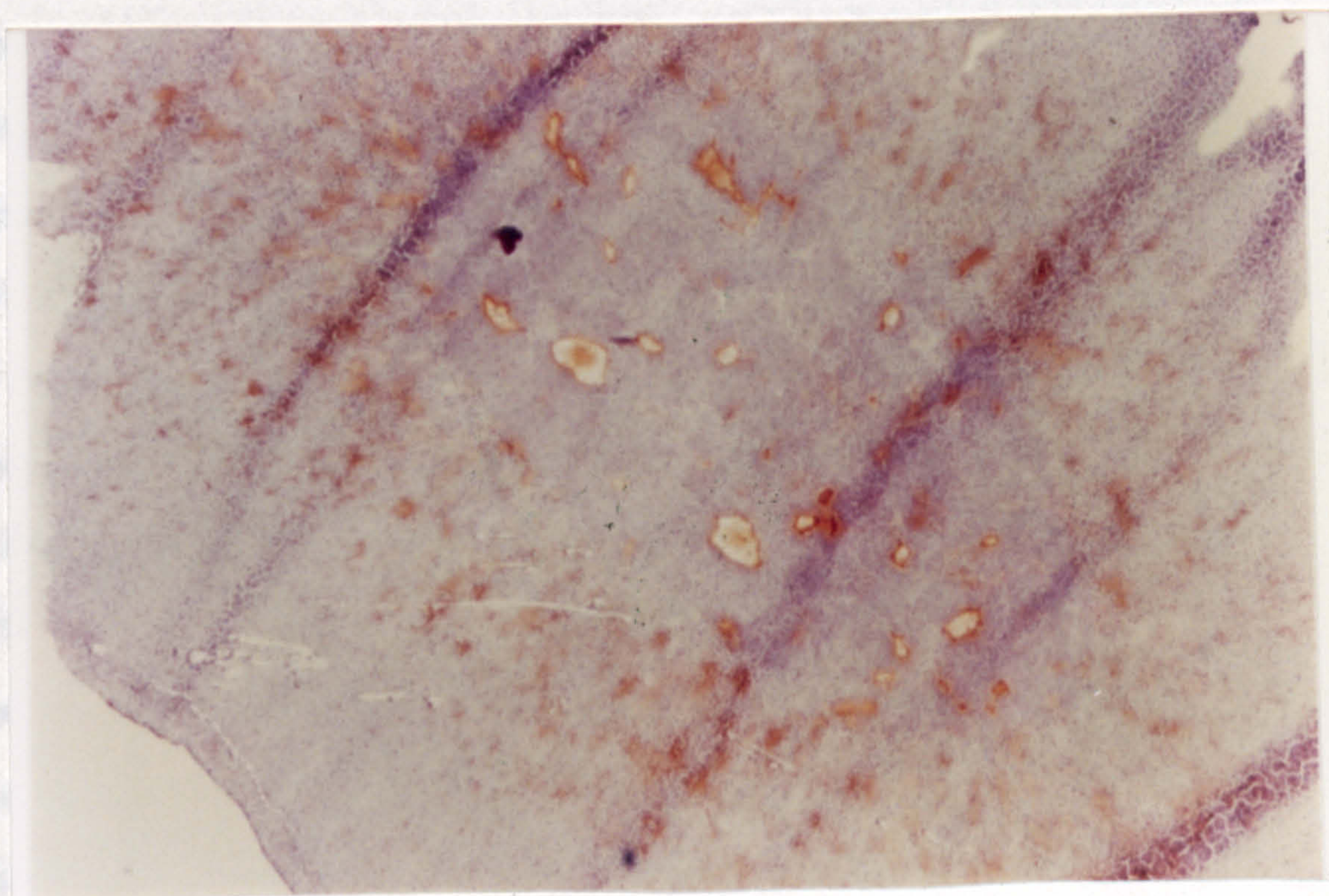


Figure 11.4

vascular drainage system which exist in the gland (see discussion). Figure 11.2 (x 400) shows the outer cortical regions stained with eosin and oil red O . The capsule is clearly distinguished as an amorphous band of fibrous tissue stained blue. Just below this region, lies the zona glomerulosa cells, distinguished by the close clusters of ovoid type cells. The haematoxylin stain reveals the large nucleus of these cells with characteristically little cytoplasm. The oil red O for steroid is present but sparsely scattered throughout the zone.

The larger area below the zona glomerulosa zone is known as the zona fasciculata, and as can be seen here, comprises much larger cells than the glomerulosa layer, with more cytoplasm per cell. Hence there is a greater abundance of lipid droplets per cell. The cells appeared to be arranged in parallel longitudinal arrangements radiating towards the centre of the gland.

Figure 11.3 taken at x800 magnification, focuses on the inner zone of the cortex and the medulla. This region consists of a narrow band of cells with large nucleus to cytoplasm ratio. Thus, there is less steroid per cell compared to the zona fasciculata and further, the cells appeared to occur in clusters anastomosing around sinusoids. This zone is known as the zona reticularis region, and impinges on the medullary zone. The cells in the medulla are much larger than the cortical cells and do not stain for lipid. They appear to have large nuclei and are arranged in dense clusters broken up by the sinusoid network.

11.8.2 DAB Staining for 5HT

Figure 11.4 shows under low power magnification (x 160) the staining pattern seen when adrenal sections were treated with the DAB stain without prior peroxide treatment. As can be seen, the characteristic brown stain is less intense in this section, compared with the brain section described below. Further, it appears to be diffusely spread throughout the adrenal section and confined to most sinusoidal regions. Note that there is no sign of any staining in the capsular region, or even the medullary cell region.

Figures 11.5 and 11.6 both were observed at x 800 magnification and show more clearly the localisation of the stain. In figure 11.5 the medullary region is stained with haematoxylin and DAB, and it clearly shows that 5HT is not to be found within the cytoplasm of medullary cells. There is however, diffuse brown staining in the regions lining the medullary sinusoids although it does not appear to be contained in any distinguishable structure.

Figure 11.6 focuses on the cortical region, and is stained with haematoxylin and DAB. Here again, the paler diffuse brown stain is restricted to the sinusoids and is not seen in any cellular structures.

Figure 11.7 shows a section of the dorsal raphe of rat brain under x 400 magnification. The section was treated with DAB stain. Within the central region and the fasciculatus longitudinali medialis, there is intense dark brown staining against a more diffuse brown stain throughout the dorsal raphe section.

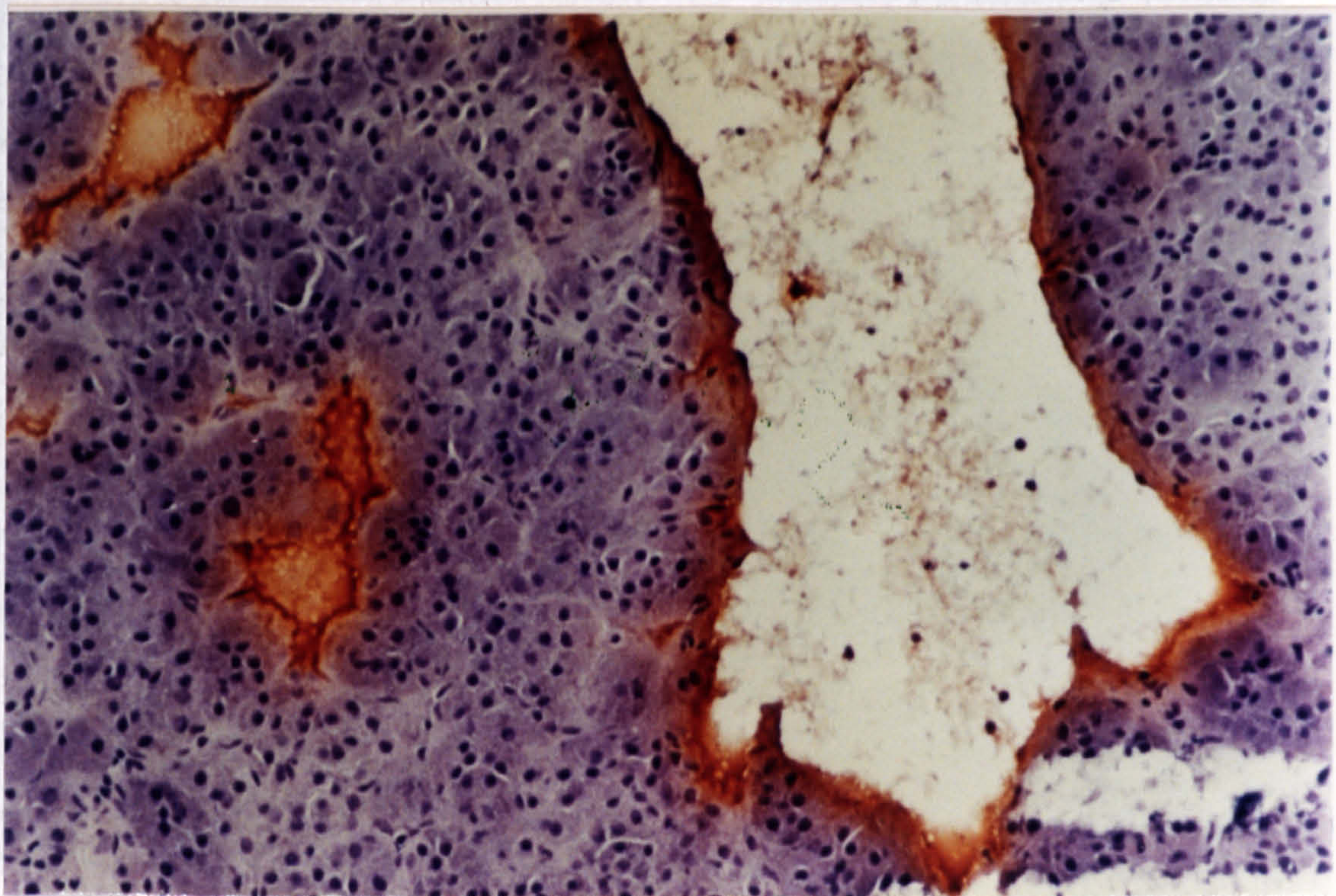


Figure 11.5

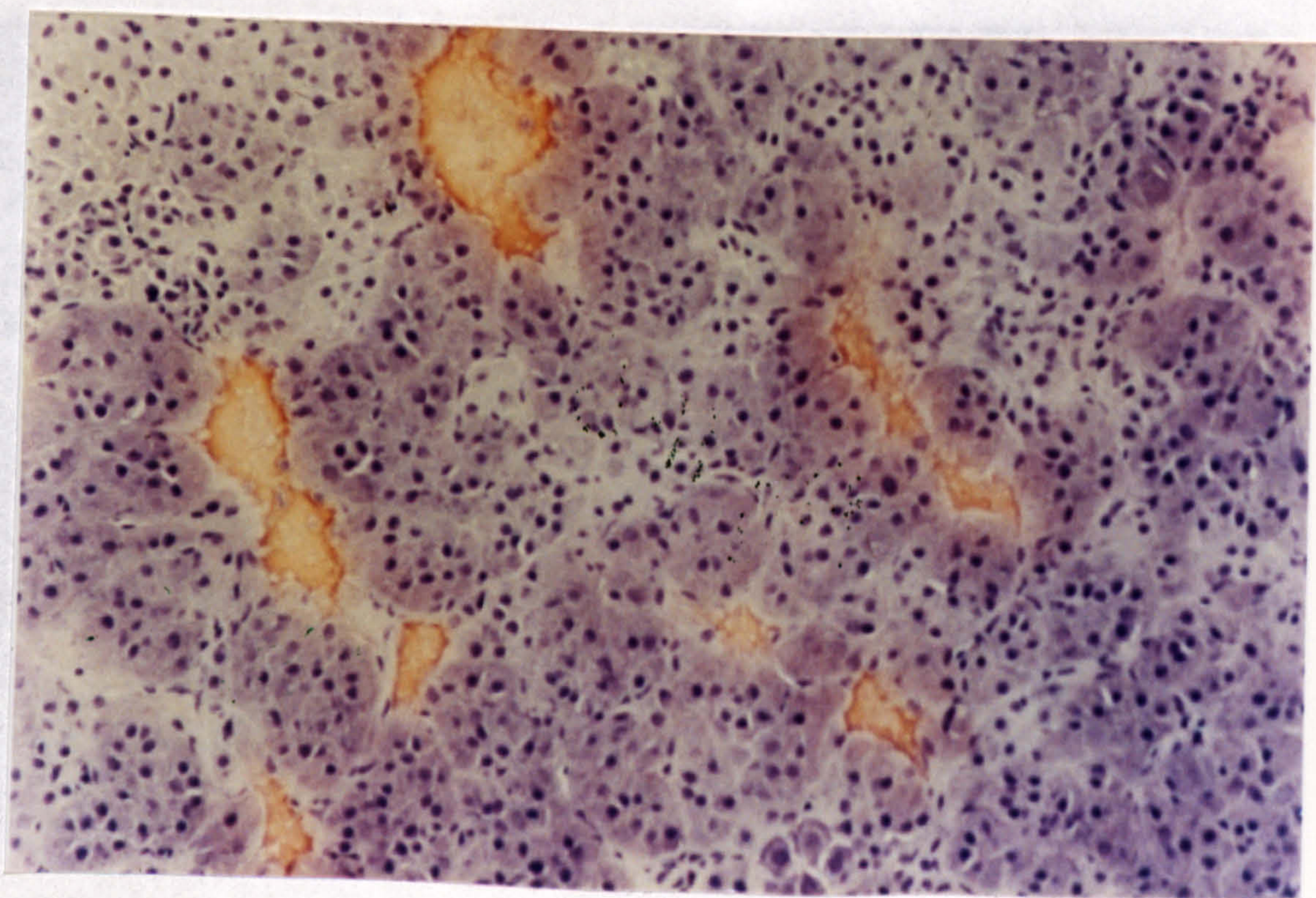


Figure 11.6

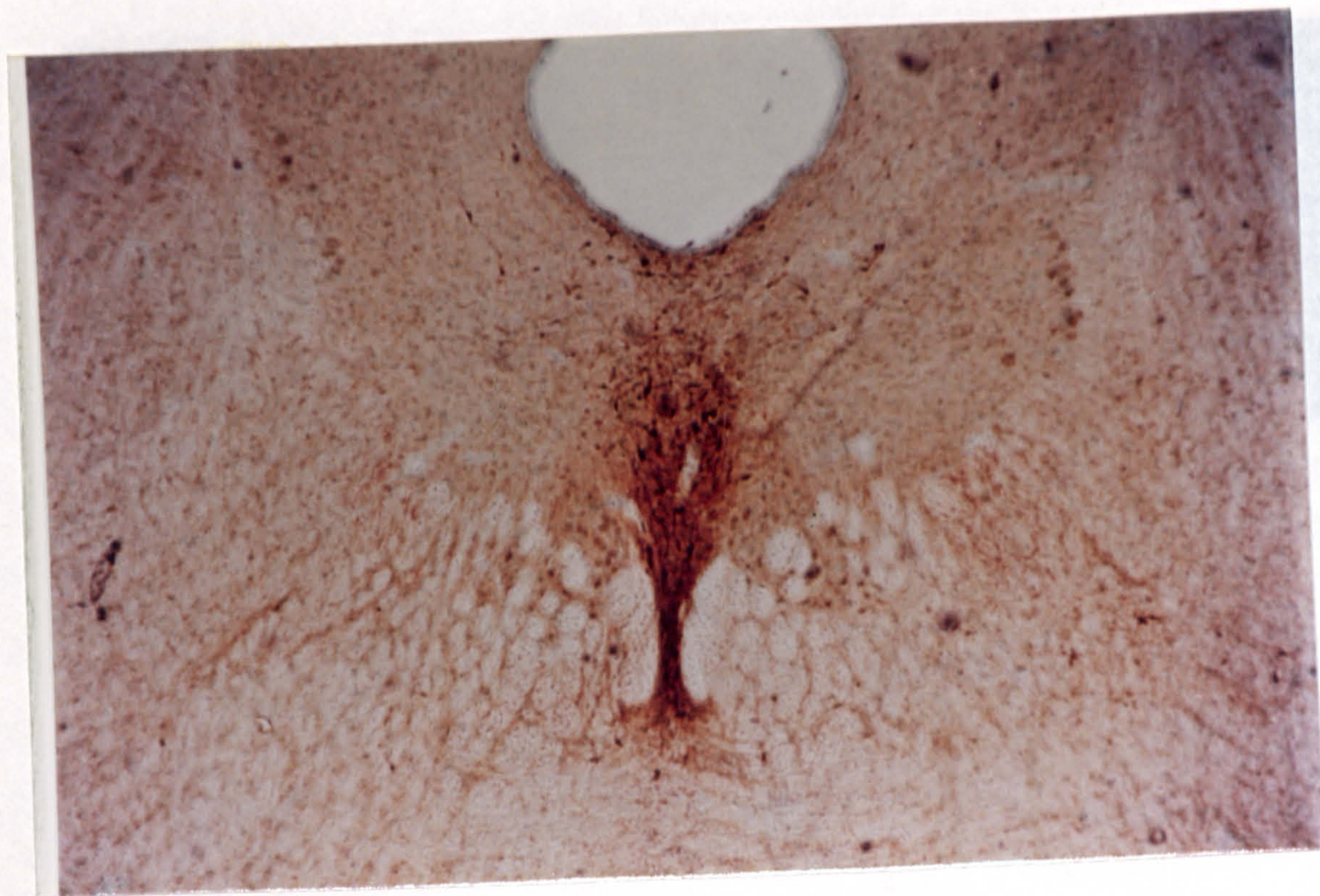


Figure 11.7

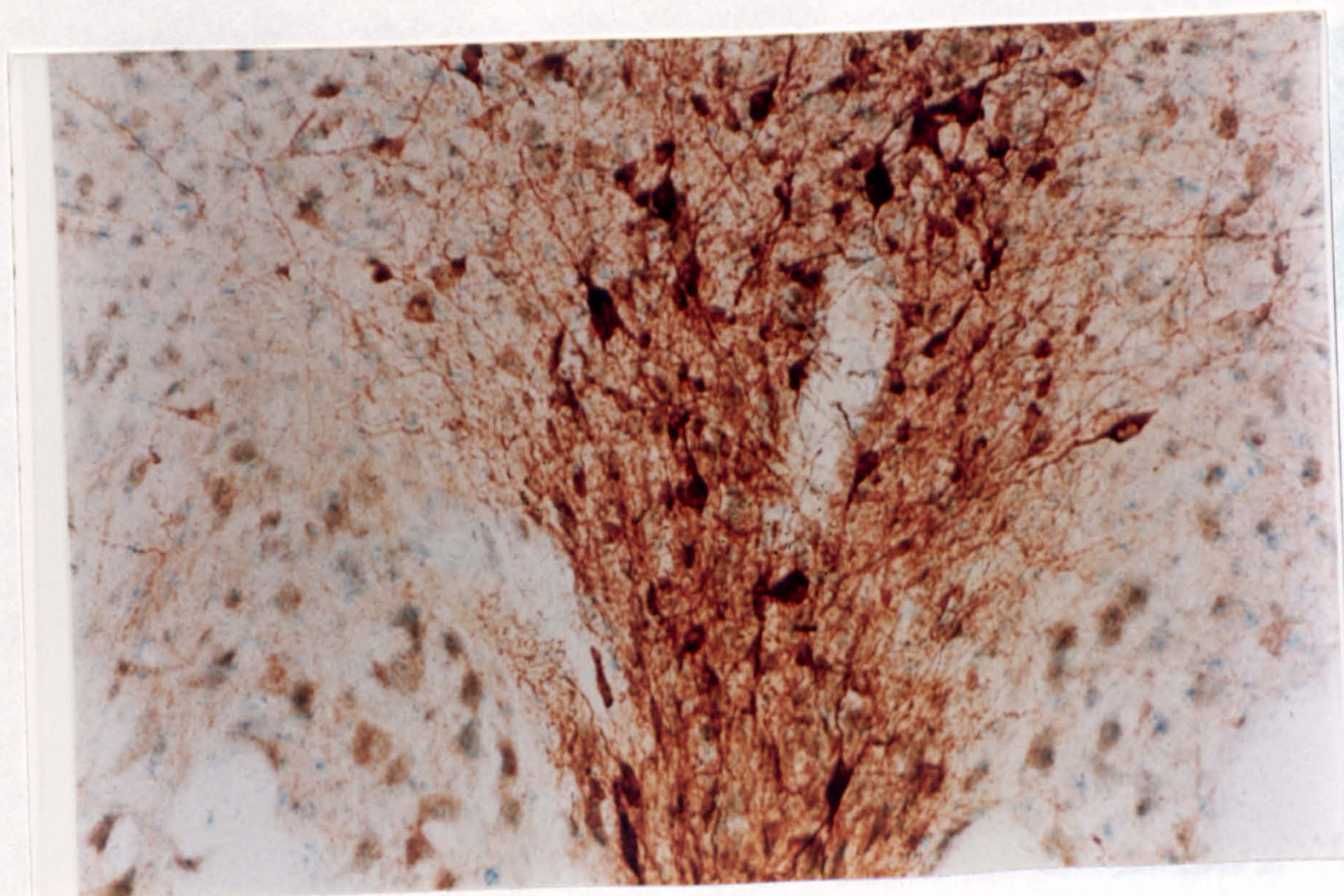


Figure 11.8

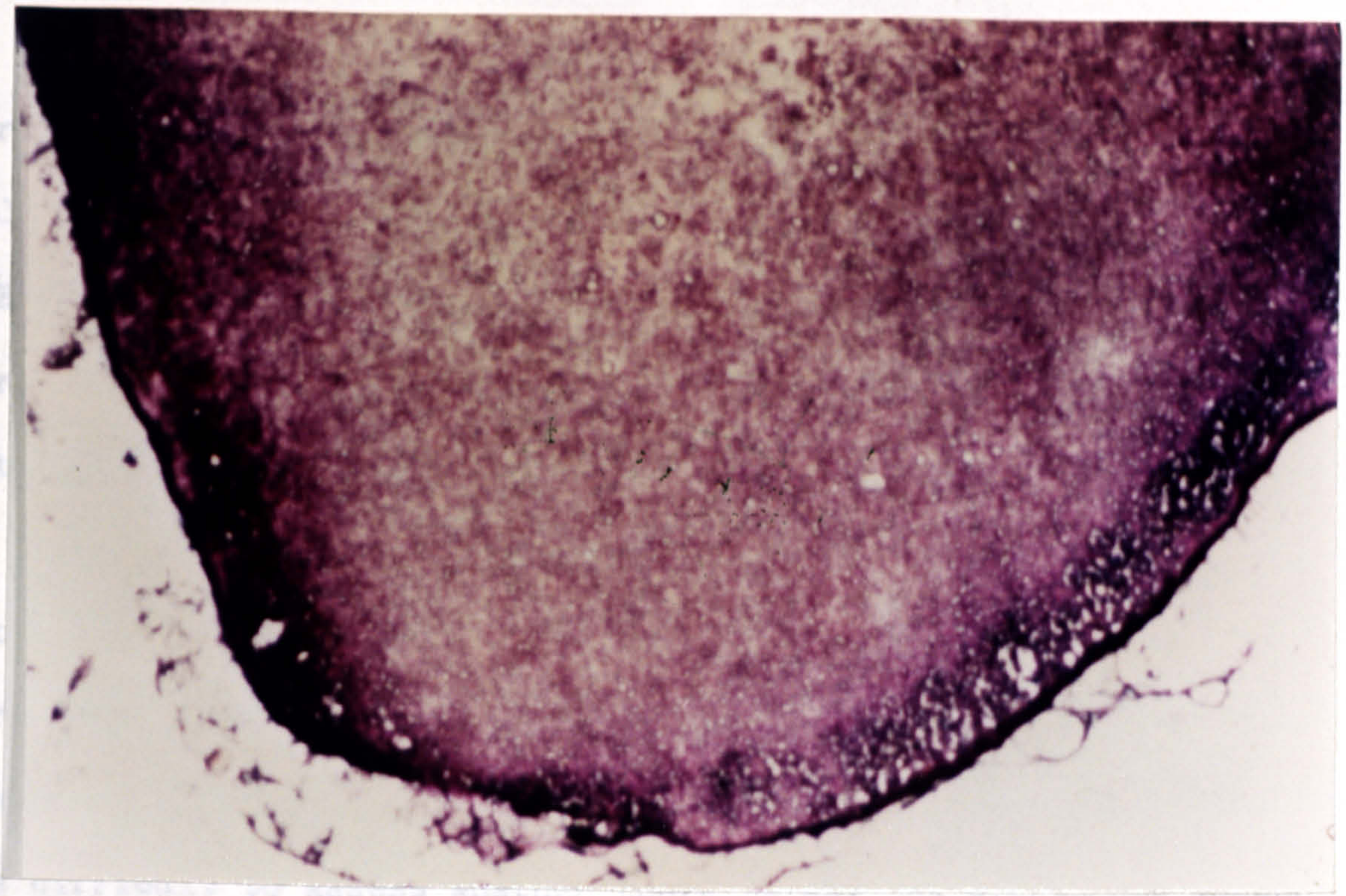


Figure 11.9

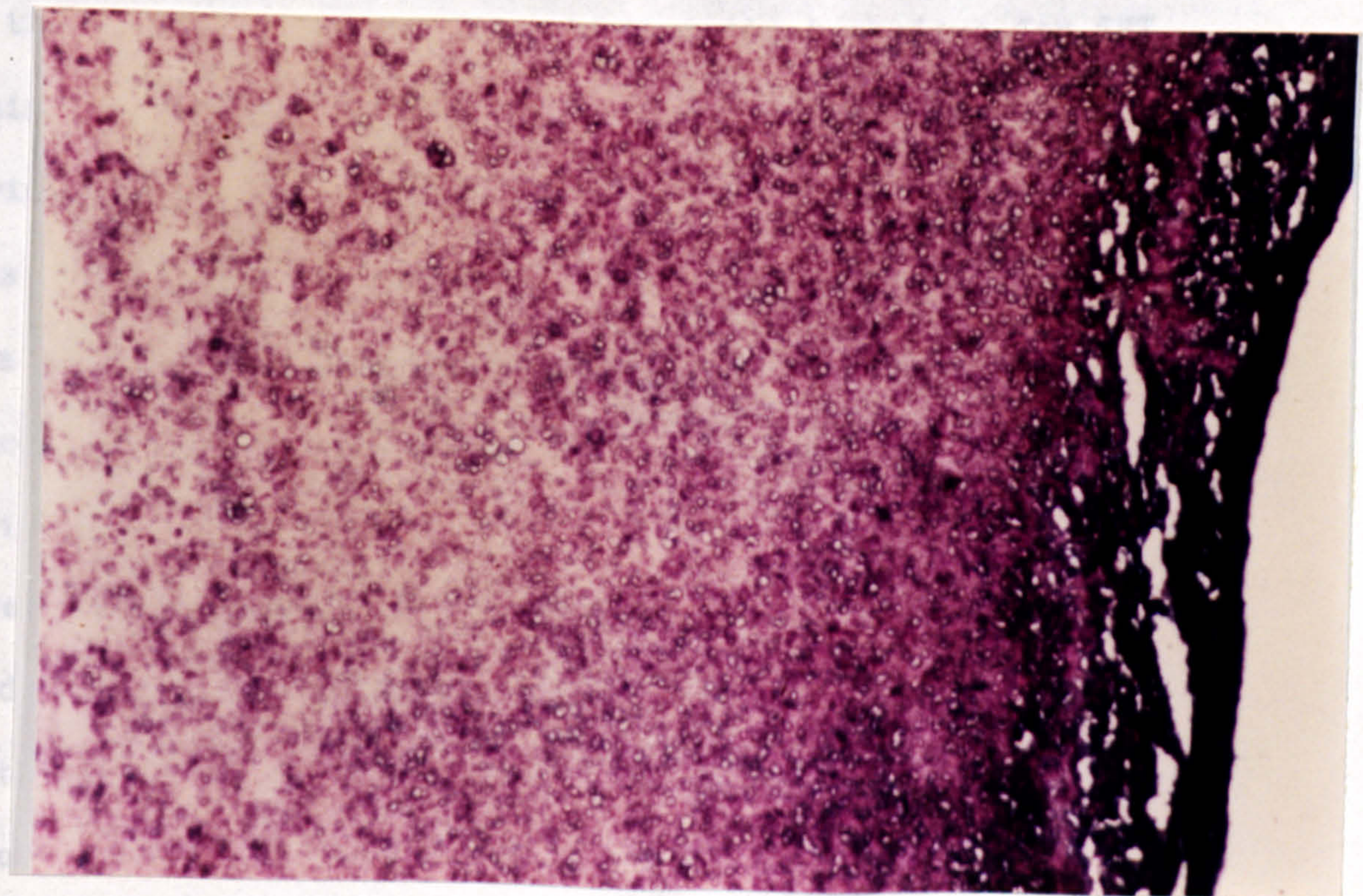


Figure 11.10

Figure 11.8 (x 1600) shows more clearly with DAB stain alone, the localisation of 5HT to the dorsal raphe region in a distinct network of 5HT-containing neurones with cytoplasmic extensions.

Figure 11.9 shows a low power adrenal section (x 400) after pre-treatment with peroxide and haematoxylin stain, counter-stained with DAB. There is no diffuse brown stain even within the sinusoidal regions.

Figure 11.10 shows the same section under x 800 magnification. There is no brown staining within the inner zones of the adrenal, however it is difficult to assess whether there is any 5HT within the deeper staining capsular region.

11.9 DISCUSSION

From these results, it can be said that the technique for 5HT staining has worked in brain tissue, thus demonstrating that in these experiments, the procedure is optimised and the reagents are viable. As has already been reported, 5HT was localised in the dorsal raphe (Buffa et al., 1980., Steinbusch et al., 1978), using sensitive immunohistochemical techniques, and was further confirmed in these experiments.

However, in the adrenal gland sections using exactly the same procedure and preparation, the 5HT was not found in any cellular structures throughout the gland. This is in contrast to the immunohistochemical observations of Verhofstadt (1983) and Holzwarth (1983). In these experiments, whole adrenals were pre-fixed with 4%

paraformaldehyde before being processed in a similar fashion to the above procedure. They were able to localise 5HT-containing cells within the rat adrenal medullary cell cytoplasm and further, they found negative staining for 5HT in nerve fibres preserved in sectioning. However, it may be worthy of note that pre-treatment of the adrenal sections with H_2O_2 without DAB, removed the diffuse brown staining originally found in untreated sections - frequently around the sinusoidal regions but sometimes intracellularly. This treatment, routinely used in brain section staining, has the effect of removing nonspecific catalytic reactions which would otherwise give less defined results. This strongly suggests that in these experiments, there was clearly no 5HT present either in the medulla or in cortical cells. Petrovic (1984) using immunohistochemical staining in the rat kidney and adrenal, was not able to localise it within the adrenal, although it was evident within the kidney. Nerve fibres which also possibly contain 5HT, were never seen under the rigours of this processing, unlike other studies (Kleitman et al., 1985., Mikhail et al., 1961,1965., Lever 1953) where nerve fibres were visualised. Endothelial cells inactivate 5HT by monoamine oxidase activity, and may also have peroxidase activity, which could have caused a similar colorimetric reaction as occurs in the presence of red blood cells, thus accounting for the dark brown staining in the sinusoid wall linings. What can be definitely concluded from these results is that at this concentration of ABC complex, there is no visible sign of 5HT-containing cellular structures in the rat adrenal, despite large quantities of 5HT (0.6-1 μ g/g wet weight - Verhofstadt et al.,

1981., Kuriyama et al., 1984., Synder et al., 1965 - see chapter 8) being found by biochemical measurement, in amounts comparable to those found in brain tissue (0.5 - 2 $\mu\text{g/g}$ (Kuriyama et al., 1984., Osborne 1982). Alternatively, 5HT may only be present in the blood and would therefore not appear in tissue sections where the slides had been 'primed'. However, this is not likely to be the complete story since the adrenal gland contains high amounts of 5HT even when perfused, thus, effectively removing blood 5HT (see Chapters 8 and 9).

No nerve fibres were visualised in any tissue sections, reducing the likelihood of locating neural 5HT within the adrenal gland.

It has already been discussed that in brain tissue and platelets, specific binding proteins exist which protect 5HT from enzymatic degradation (Tamir et al., 1980). However the protein structures in brain for binding 5HT, differ from the platelet glycoproteins and albumin. In this study, the possibility that 5HT is bound to a protein molecule different from the one recognised by the 5HT antibody in the brain or platelets, is an alternative explanation for the negative results.

From these observations, it can only be said that 5HT could not be found in any region of the adrenal parenchyma, but the procedure was optimal for brain staining of 5HT. Further, it was not possible to visualise serotonergic nerve fibres under the rigours of this procedural technique.

There exists the possibility that 5HT, if an endogenous amine of rat tissue, could indeed have been stored, but like for example in the

eye structure of Aplysia, where 5HT is measured biochemically but not visualised histochemically (Lubosky-Moor et al., 1976) could not be visualised. In the studies carried out by Lubosky-Moor, fluorescence techniques were used to visualise catecholamines in several tissues of the Aplysia, and 5HT was found in the heart and neural structures, but not in the eye, although it was measured biochemically with levels as high as 0.05 µg/g tissue (Corrent et al., 1978). These levels are at the lower end of the measured levels in the adrenal studies (Chapter 8), and despite a more sensitive antibody detection method for 5HT, it could, as indicated above, be due to a different type of binding association in adrenal tissue compared with other tissues. These results still beg the question, whether 5HT is an endogenous amine of the rat adrenal, or a blood-borne factor. Under the rigours of this technique, it favours the latter conclusion but would require considerable time in optimising the technique, to support these initial findings.

Chapter 12

SUMMARY AND CONCLUSIONS

The accumulated data goes some way in describing a physiological role for serotonin in the control of aldosterone biosynthesis in the rat model.

The drug antagonism studies, although requiring further investigation, tend to suggest a 5HT-response which is only loosely coupled to cAMP-stimulated steroidogenesis and then, only measured at higher doses of serotonin. More interestingly, the serotonergic interaction of the antagonists with angiotensin II and ACTH may indicate a 5HT interaction and potentiation of the steroid response to important physiological stimuli.

However, the data in Chapter 7 does not exclude the possibility that 5HT is interacting through its own specific receptor - 5HT₁ as these studies suggest - and is coupled to both cAMP and a Ca^{2+} and/or the PI system as discussed in other systems - in the second messenger response, which would result in complex but feasible interaction and potentiation of the steroid response to ACTH and AII. The Taitts (1972) studied the potentiating effects of 5HT on the potassium dose response curves, and suggested that at higher doses of K^+ i.e. $>8.4 \text{ mM}$, the steroid response was maximal even in the presence of 10^{-6} M 5HT, a dose known to increase cAMP levels, and so the two stimuli worked through the same second messenger; in contrast at lower doses of K^+ , the effects of K^+ were potentiated in the presence of 10^{-6} M 5HT, indicating a separate mechanism of action. Kojima's study (1985) indicates that at 8 mM , calcium has important effects on

inducing the transient phase increase in aldosterone secretion with cAMP being important in the sustained response; thus by extrapolation 5HT at 10^{-6} M may be acting predominantly through the cAMP mechanism of stimulation. However, to verify the theory of these results would require looking at the potentiating effect of several doses of 5HT with various doses of the other stimuli and more readily, at the second messenger systems for 5HT alone at increasing doses, to discern what second messengers are involved in its mechanism of action. Further, the antagonism studies using 5HT antagonists rather than the 5HT molecule in steroid experiments, cannot be extrapolated to mean that they are having effects beyond a putative 5HT receptor site, i.e. at the intracellular level. Moreover, the antagonists themselves have specific pharmacological profiles and may exert effects on the plasma membrane which are separate from their binding to putative 5HT receptor sites. In the salt studies, 5HT levels measured in serum, were not significantly altered, but this does not rule out the possibility that 5HT turnover may be altered. Further investigations measuring 5HT levels on a number of platelets basis would yield more information in altered sodium status and define if any, a role for 5HT in the changing basal and stimulated steroid outputs in the adrenal gland.

Within the gland itself, 5HT was measurable in relatively high amounts per unit weight of tissue. Although levels did not significantly alter - this can be interpreted in several ways: 1. the measurement did not take into account the rate of turnover within the tissue which could have changed in altered salt status - enzyme activity and particularly tryptophan hydroxylase activity would need to be measured to demonstrate this; 2. 5HT in the adrenal gland is not involved in physiological changes occurring in vivo e.g. salt status. This latter theory would argue against an important paracrine role for 5HT in salt balance, in contrast to the suggestion that dopamine may be involved in the blunted response to AII in high salt status (Aguilera 1984). However, the biochemical measurements of 5HT in the adrenal gland are interesting in that large amounts appear to be present in the inner cortex and medulla. To further quantify and determine the derivation of 5HT, similar determinations for norepinephrine (NE) and epinephrine (E) ratios within the regions of the adrenal gland compared with 5HT:NE:E ratios, should be determined. In this way, the ratios if constant, would define the site of origin for 5HT, as previously described for dopamine since it has already been established that NE and E are present within the adrenal in constant ratios (Hannah 1984). As indicated in Chapter 11, the negative staining for 5HT may be a consequence of its binding to an inaccessible protein. Indeed Kuriyama (1984) and Bertler (1960), have suggested that 5HT in the medulla may be bound within epinephrine containing chromaffin granules, but may be under different physiological control. This has

been demonstrated in, for example, the rat model when 5HT was selectively retained in the chromaffin granules of stressed rats although within the same granules, epinephrine was released (Kuriyama et al., 1984).

The metabolism studies using isolated glomerulosa and inner cortical and medullary cells, also indicated a greater storage of 5HT within the inner adrenal regions, and rapid metabolism in the zona glomerulosa. Osborne (1982), stipulated that a neurotransmitter role is considered if the compound is stored within the tissue. However with immunohistochemical staining, serotonergic nerve fibres were not seen. Alternatively, another suggested role for biogenic amines in a paracrine capacity, is captured in the idea postulated by Green (1962) in which mast cells may release their store of 5HT (5HT is found in rat and mice mast cells), within the adrenal tissue, and be taken up by chromaffin granules; thus, exogenous 5HT may be derived from this source. A similar study using exogenous labelled 5HT was described in the rat vas deferens (Thoa et al., 1969) and here it was suggested that sympathetic norepinephrine containing nerve endings were responsible for the uptake of exogenous 5HT. Rosenkrantz in 1960, also postulated a role for platelet-derived 5HT in the adrenal gland, particularly during haemorrhage, when damaged platelets within the gland accumulate and would release exogenous 5HT which could: 1. enter the chromaffin cells, or; 2. directly stimulate aldosterone biosynthesis - an important homeostatic mechanism in this state. Catecholamines as described in Chapter 2, have some influence on steroidogenesis, at least in vitro, particularly in

relation to the effects of dopamine in the adrenal. In a similar fashion, the catecholamines:5HT ratios within the gland, may influence and facilitate the 5HT-mediated steroidogenic response in vivo, compared to the desensitising effect which catecholamines have on the dopamine-aldosterone response of cultured zona glomerulosa cells (De Lean et al., 1984b).

Overall therefore, a fine control mechanism of catecholamines on the 5HT and dopamine-steroidogenic response may exist, which is determined by the physiological status.

The administration of precursors are interesting in that there appears to be an effect in vivo which is only apparent when the adrenal cells are removed from other physiological influences, such as circulating AII and ACTH, and incubated in vitro. As indicated in the discussion section of Chapter 8, a central role for 5HT, indirectly affecting other factors such as ACTH, perhaps acutely, must not be excluded. Connors and Rosenkrantz (1962) indicated in their work, that administration of 5HT decreases adrenal ascorbic acid levels in the same way that ACTH administration also has the same effect. This could be due to direct central release of ACTH or a more direct effect at the level of the adrenal. The reported high levels of L-aromatic amino acid decarboxylase activity in the adrenal gland (Vaccari et al., 1977) make it possible that administration of the precursor of 5HT, 5HTP, allows local conversion within the gland to 5HT, which is responsible for increasing hydroxylating activity of the steroidogenic enzymes, and thus elevating basal steroid levels secreted by the adrenal, in 5HTP-treated rats. Connors and

Rosencrantz (1962) observed an increase in hydroxylating capacity of the adrenal using isolated rabbit adrenal slices incubated with 5HT - the converse may be true in PCPA-treated rats.

Histochemical observations on the adrenal were not made with PCPA/5HTP treatment, which may also have elucidated the effects on steroid biosynthetic capacity being due to gross morphological changes in the gland. In these experiments 5HTP administration increased adrenal steroidogenic capacity, and unless adrenal size increased, 5HT may have directly increased steroid enzyme capacity. Alternatively, increased 5HT levels may have increased central AII and/or peripheral AII levels acutely, but chronic studies measuring plasma concentrations of AII did not show any change; central AII effects may have been responsible through neural adrenal connections, for increasing basal steroid levels. The converse situation in which steroids increase 5HT biosynthesis is well documented (Azmitra et al., 1974) and so the possibility exists that administered 5HTP acted centrally and increased the release of pituitary factors involved in increased steroid biosynthesis in the in vitro studies. Further investigations to exclude a nonspecific effect on steroidogenic capacity in all regions of the adrenal would be needed, using isolated zona fasciculata cells as well as glomerulosa cells. The investigations delineate a stimulatory role for 5HT in the zona glomerulosa but exogenously-derived 5HT from platelet and/or mast cells which may be temporarily stored in the medullary region, make it more than likely that this biogenic amine is only a potentiator of the major physiological stimuli. In particular, the drug antagonism

studies suggest that the mechanism of action for 5HT may not be solely through 5HT receptors (yet to be defined in the zona glomerulosa) but act through an interaction with ACTH- and AII-stimulated steroidogenesis at the post-receptor site. To postulate, conditions in which 5HT may be involved in modulating the aldosterone response may include haemorrhagic states; in altered salt status further investigations in altered salt diets, may indicate a role for 5HT in changing the adrenal sensitivity to AII, perhaps the most important physiological regulator of aldosterone in the rat and Man, and therefore by extrapolation indicate a significant role for 5HT. The field of research into 5HT in the adrenal is only now beginning to unfold.

REFERENCES

- Achilli, G., Perego, C., Ponzio, F. and Algeri, S. (1983) Simultaneous determination of dopamine, norepinephrine, serotonin and their metabolites in several rat brain areas by high performance liquid chromatography with electrochemical detection. *Res. Commun. Pathol. Pharmacol.* 40 (1):67-72.
- Aguilera G., White A. and Marusic E.T. (1975) The importance of sodium balance in the effect of AII on aldosterone production. *Acta Endocrinol (Kbh)* 80:104-13
- Aguilera G. and Catt K.J. (1978a) Regulation of aldosterone secretion by the renin-angiotensin system during sodium restriction in rats. *Proc. Natl Acad. Sci. USA* 75(8):4057-4061
- Aguilera G., Hauger R.L. and Catt K.J. (1978b) Control of aldosterone secretion during sodium restriction: Adrenal receptor regulation and increased adrenal sensitivity to angiotensin II. *Proc. Natl Acad. Sci. USA* 75:975-979
- Aguilera G. and Catt K.J. (1979a) Loci of action of regulators of aldosterone biosynthesis in isolated zona glomerulosa cells. *Endocrinology* 104(4) :1046-1052.
- Aguilera, G., Capponi, A., Banhal, A., Fujita, K., Hauger, R. and Catt, K.J. (1979b) Metabolism and biological activity of AII and des-Asp¹-AII in isolated adrenal zona glomerulosa cells. *Endocrinology* 104:1279-1285.
- Aguilera G., Menard, R.H. and Catt K.J. (1980) Regulatory actions of angiotensin II on receptors and steroidogenic enzymes in adrenal zona glomerulosa cells. *Endocrinology* 107:55-60
- Aguilera G., Harwood J.P. and Catt K.J. (1981) Somatostatin modulates the effects of angiotensin II in the adrenal zona glomerulosa. *Nature* 292:262-263
- Aguilera G., Parker D.S. and Catt K.J. (1982) Characterisation of somatostatin receptors in the rat zona glomerulosa zone. *Endocrinology* 111:1376-1384
- Aguilera G and Catt K.J. (1984) Dopaminergic modulation of aldosterone secretion in the rat. *Endocrinology* 114(1):776-781
- Aikawa, T., Hirose, T., Matsumoto, I. and Suzuki, T. (1979) Secretion of aldosterone in response to histamine in hypophysectomised-nephrectomised dogs. *J. Endocrinol.* 81:325-330.

Albano, J.D.M., Brown, B.L., Ekins R.P., Tait S.A.S. and Tait J.F. (1974) The effects of potassium, serotonin, ACTH and AII on the concentration of adenosine 3',5' cyclic monophosphate in suspensions of dispersed rat adrenal zona glomerulosa and zona fasciculata cells. *Biochem J.* 142:391-400

Albrecht P., Visscher, M.B. and Bittner J.J. and Halberg, F. (1956) Daily changes in serotonin concentrations in mouse brain. *Proc. Soc. Biol. Med.* 92:703-709

Al-Dujaili, E.A.S., Boscaro, M., Espiner, E.A. and Edwards, C.R.W. (1980) In vitro and in vivo effects of indoleamines on aldosterone biosynthesis in the rat. Abstr 400, Proceedings of the VI International Congress of Endocrinology, Melbourne, Australia.

Al-Dujaili, E.A.S., Hope J., Estivariz F.E., Lowry P.J. and Edwards C.R.W. (1981a) Circulating human pituitary pro gamma MSH enhances adrenal response to ACTH. *Nature* 291:156-157

Al-Dujaili, E.A.S., Williams, B.C. and Edwards, C.R.W. (1981b) The development and application of a direct radioimmunoassay for corticosterone. *Steroids* 37(2):157-176.

Al-Dujail, E.A.S. and Edwards, C.R.W. (1981c) Optimisation of a direct radioimmunoassay for plasma aldosterone. *J. Steroid Biochem.* 14:481-487

Al-Dujaili, E.A.S., Boscaro M. and Edwards C.R.W. (1982) An in vitro stimulatory effect of indoleamines on aldosterone biosynthesis in the rat. *J. Steroid Biochem.* 17:351-355

Alexander, R.W., Gill J.R., Yanabe H., Lovenberg W. and Keiser H.R. (1974) Effects of dietary sodium and of acute saline infusion on the interrelationship between dopamine excretion and adrenergic activity in Man. *J. Clin. Invest.* 54:194-200

Allgren, R.L., Kyncl, M.M. and Ciaranello, R.D. (1985) Pharmacological characterization of solubilised 5-HT₁ serotonin binding sites from bovine brain. *Brain Res.* 348 (1):77-85.

Ames, R.P., Borkowski, A.J. and Sicinski, A.M. (1965) Prolonged infusions of AII and norepinephrine on blood pressure, electrolyte balance, aldosterone and cortisol secretion in normal man and cirrhosis and ascites. *J. Clin. Invest.* 44:1171-1186

Anderson, C.H., McCally and Farrell G.L. (1959) The effects of atrial stretch on aldosterone secretion. *Endocrinology* 64:202-207

Ang, V.T.Y. and Jenkins, J.S. (1984) Neurophysial hormones in the adrenal medulla. *Endocrinology* 58(4):688-691

Atarashi, K., Mulrow, P.J., Franco-Saenz, R., Snajdar, R. and Rapp, J. (1984) Inhibition of aldosterone production by an atrial extract. *Science* 224:992-994

Azmitra, E.C. and McEwen B.S.(1974) Adrenocortical influence on rat brain tryptophan hydroxylase activity. *Brain Res.* 78:291-302

Bakker, R.F.M. and de Wied, D. (1960) The effect of serum on steroid formation rate in vitro on rat adrenals. *Acta Physiol. Pharmacol.* 9:123-125.

Balla, T., Enyedi P., Spat, A. and Antoni, F.A. (1985) Pressor type vasopressin receptors in the adrenal cortex: properties of bindings, effects on phosphoinositide metabolism and aldosterone secretion. *Endocrinology* 117(1):421-423

Barath, P. and Csaba, G. (1973) Effect of pinealectomy on the tritiated 5HT uptake of the adrenal medulla. *Horm. Metab Res* 5:466-470.

Baudouin-Legros, M., Le Quan-Bui, K.H., Guicheney, P., Kamal, L.A. and Meyer, P. (1985) Platelet serotonin in essential hypertension and in mental depression. *J. Cardiovasc. Pharmacol.* 7 (Supp 7):S12-S14

Baumann, K. and Muller, J. (1972) Effect of potassium intake on the final steps of aldosterone biosynthesis in the rat. I 18 hydroxylation and 11-hydroxy dehydrogenation. *Acta Endocrinol.* (Copenh) 69:701-717

Baumann, J.B., Eberle, A.N., Christen, E., Ruch, W. and Girard, J. (1986) Steroidogenic activity of highly potent melanotropic peptides in the adrenal cortex of the rat. *Acta Endocrinol.* 113 (3) :396-402

Baumber, J.S., Davis, J.O. Johnson, J.A. and Witty, R.T. (1971) Increased adrenocortical potassium in association with increased biosynthesis of aldosterone. *Am. J.Physiol.* 220:1094-1099

Bennett, J.P. and Snyder, S.H. (1976) Serotonin and lysergic acid diethylamide binding in rat brain membranes: relationship to postsynaptic 5HT receptor. *Mol. Pharmacol.* 12:373-389

Berod, A., Hartman, B.K. and Pujol J.F. (1981) Importance of fixation in immunohistochemistry: use of formaldehyde solutions at variable pH for the localisation of tyrosine hydroxylase. *J. Histochem. Cytochem.* 29 (7):844-850

Berridge, M.J. and Fain, J.N. (1979) Inhibition of phosphoinositol synthesis and the inactivation of calcium entry after prolonged exposure of the blowfly salivary gland to 5HT. *Biochem. J.* 178(1):59-69

Berridge, J.B. (1980) Receptors and calcium signalling. Trends Pharmacol. Sci. 1 (15):420-424

Bertler, A., Rosenberg, A.M. and Rosenberg, E. (1960) In vivo uptake of dopamine and serotonin by adrenal medullary granules. Experientia 16(9):418-419

Bevilacqua, M., Vago, T., Sorza, D. and Norbiato, G. (1982) Characterisation of dopamine receptors by [³H]-ADTN binding in calf adrenal zona glomerulosa. Biochem. Biophys. Res. Commun. 108:1661-1669

Biglieri, E.G. (1984) The pituitary and idiopathic hypertension. N. Engl J. Med. 311(2):120-121

Bing, R.E. and Schulster, D. (1977) Steroidogenesis in isolated rat adrenal glomerulosa cells: response to physiological concentrations of AII and effects of potassium, 5HT and [Sar¹, Ala⁸]-AII. J. Endocrinol. 74:261-272

Birkhauser, M., Kiondel, A. and Vallotton, M.B. (1979) Bromocriptine induced modulation of plasma aldosterone response to acute stimulations. Acta Endocrinol. 91:294-302

Birmingham, M.K., Elliot, F.H. and Valere, P.H.L. (1953) The need for the presence of Ca²⁺ for the stimulation in vitro of rat adrenal gland by ACTH. Endocrinology 53:687-689

Black, J.L., French, R.J. and Mylecharane, E.J. (1981) Receptor mechanisms for 5-hydroxytryptamine in rabbit arteries. Br. J. Pharmacol. 74:619-626

Blair-West, J.R., Coghlan, J.P., Denton, D.A., Goding, J.R., Munro, J.A., Peterson, R.E. and Wintour, M. (1962) Humoral stimulation of adrenal cortical secretion. J. Clin. Invest. 41:1606-1627.

Blair-West, J.R., Brodie, A., Coghlan, J.P., Denton, D.A., Flood, C., Goding, J.R. et al., (1970) Studies on the biosynthesis of aldosterone using the sheep adrenal transpant I Effects of sodium depletion on the conversion of corticosterone to aldosterone. J. Endocrinol. 46:453-476.

Blanchard, J. (1981) Evaluation of the relative efficacy of various techniques for deproteinising plasma samples prior to HPLC. J. Chromat. 226:455-460

Bosacaro, M., E.A.S. Al-Dujaili and Edwards, C.R.W. (1979) In vitro studies on the mechanism of inhibition by bromocriptine, lisuride and metergoline by AII-induced aldosterone release. Abstr 70, Acta Endocrinol, Munich, Germany.

Boyd, J.E., Palmore, W.P. and Mulrow P.J. (1971) Role of potassium in the control of aldosterone in the rat. *Endocrinology* 88:556-565

Braley, L. and Williams, G.H. (1977) Rat adrenal cell sensitivity to AII, -1-24 ACTH and potassium: a comparative study. *Am. J. Physiol.* 233:402E-406E

Braley, L., Menachery, A., Brown E. and Williams, G.H. (1984) The effects of extracellular potassium and AII on cytosolic calcium and steroidogenesis in adrenal glomerulosa cells. *Biochem. Biophys. Res. Commun.* 123(2):810-815

Bristow, A.F., Gleed, C. and Fauchere, J. (1980) Effects of adrenocorticotropin (ACTH) analogues on steroidogenesis and cyclic AMP in rat adrenocortical cells. Evidence for two different steroidogenically responsive receptors. *Biochem. J.* 186:599-603

Brown, R.D., Wisgerhof, M. Nai-Saing, Jiang., Kao, P. and Hegstad, R. (1977) Effect of metoclopramide on the secretion and metabolism of aldosterone in Man. *J. Clin. Endocrinol. Metab.* 52(5) :1014-1018

Brown, R.D., Wisgerhof, M. Carpenter, P.C., Brown, G., Jiang, N.S., Kao, P. and Hegstad, R. (1979) Adrenal sensitivity to angiotensin II and undiscovered aldosterone stimulating factors in hypertension. *J. Steroid Biochem.* 11:1043-1050

Brown, B.L. (1982) The role of cyclic nucleotides and calcium in adrenocortical function (Chapter 30) in the *Handbook of Experimental Pharmacology* 58/II (Kebabian J.W. and J.A. Nathanson eds)

Buckley, D.I., Yamashio, D. and Ramachandran, J. (1981) Synthesis of a corticotropin analogue that retains the full biological activity after iodination. *Endocrinology* 109(1):5-9

Buffa, R., Crivelli, O., Lavarinin, C., Sessa, F., Verme, G. and Solcia, F. (1980) Immunohistochemistry of brain 5-Hydroxytryptamine. *Histochemistry* 68(9):9-15

Burwell, L.R., Davis, W.W. and Bartter, F.C. (1969) Studies on the loci of action of stimuli to the biogenesis of aldosterone. *Proc. Roy. Soc. Med.* 62:1254-1257.

Campbell, D.J., Mendelsohn, F.A.O., Adam, W.R. and Funder, J.W. (1981) Metoclopramide does not elevate aldosterone in the rat. *Endocrinology* 109(5):1484-1491

Campbell, D.J. (1982) Effect of composition of medium on aldosterone and corticosterone production by isolated zona glomerulosa cells. *J. Endocrinol.* 94:211-224

Cappuccio, F.P., Markandu, N.D., Buckley, M.G., Shore, A.C., Booker, J. and MacGregor, G.A. (1986) Changes in the plasma levels of immunoreactive atrial natriuretic peptide during mineralocorticoid 'escape' in Man. Abstract 7, Serona Symposium (Mannheim, Germany)

Caraballeria, A. and Venning, E.M. (1964) Conversion of steroids by chromaffin tissue. I Studies with a pheochromocytoma. Steroids 4:329-350

Carey, R.M., Darracott, V.J.R., Peach, M.J. and Ayers, C.R. (1978) Activity of [des-aspartyl]-AII and AII in Man: differences in blood pressure and adrenocortical response during normal and low sodium intake. J. Clin. Invest. 61:20-31.

Carey, R.M., Ayers, C.R., Vaughan, J.R., Peach, M.J. and Herf, S.M. (1979) Activity of [des-aspartmyl]-angiotensin II in primary aldosteronism. J. Clin. Invest. 63:718-735.

Carey, R.M., Thorner, M.O. and Ortt, E.M. (1980) Dopaminergic inhibition of metoclopramide-induced aldosterone secretion in Man. Dissociation of response to dopamine and bromocriptine. J. Clin. Invest. 66:10-18

Carey, R.M., VanLoom, G.R., Baines, A.D. and Ortt, E.M. (1981) Decreased plasma and urinary dopamine during dietary sodium depletion in man. J. Clin. Endocrinol. Metab. 52: 903-909.

Carey, R.M. (1982) Acute dopaminergic inhibition of aldosterone secretion is independent of AII and ACTH. J. Clin. Endocrinol. Metab. 54(2):463-469

Cascio, C.S. and Kellar, K.J. (1982) Tetrahydro-beta-carbolines affinities for tryptamine and serotonergic binding sites. Neuropharmacology 21(11):1219-1221.

Chabot, J.G. Walker, P. and Pelletier, G. (1986) Distribution of epidermal growth factor binding sites in the adult rat adrenal gland by light microscope autoradiography. Acta Endocrinol. 113 (3):391-396

Challis, J.R.G. and Torosin, J.D. (1977) Is alpha MSH a trophic hormone to adrenal function in the foetus? Nature 269:818-819

Chand, N. (1981) Reactivity of isolated trachea, bronchus and lung strip of cats to carbachol, 5-hydroxytryptamine and histamine: evidence for the existence of methysergide sensitive receptors. Br. J. Pharmacol. 73:853-857

Chartier, L., Schiffre, E., Thibault, G. and Garcia, R. (1984) ANF inhibits the stimulation of aldosterone secretion by AII, ACTH and potassium in vitro and AII induced steroidogenesis in vivo. Endocrinology 115(5): 2026-2028

- Cheitlin, R., Buckley, D.I. and Ramachandran, J. (1985) The role of extracellular calcium in corticotropin-stimulated steroidogenesis. *J. Biol. Chem.* 260 (9):5323-5327
- Cheng, J.B. and Shibata, S. (1980) Pressor response to 5-hydroxytryptamine, norepinephrine and KCl in the perfused hindquarter preparation from the spontaneously hypertensive rat. *J. Pharmacol. Exp. Ther.* 214:488-495
- Coghlan, J.P., Blair-West, J.R. Denton D.A., Fei, D.T., Fernley, R.T. Hardy, K.J. et al., (1979) Control of aldosterone secretion. *J. Endocrinol.* 81:55P-67P
- Cohen, M.L., Fuller, R.W. and Wiley, K.S. (1981) Evidence for 5HT₂ receptors mediating contraction in vascular smooth muscle. *J. Pharmacol. Exp. Ther.* 218 (2):421-425.
- Cohen, M. L. and Wittenauer, L.A. (1985) Relationship between serotonin and tryptamine receptors in rat stomach fundus. *J. Pharmacol. Exper. Ther.* 233(1):75-79
- Conn, J.P. and Sanders-Bush, E. (1986) Biochemical characterisation of serotonin stimulated phosphoinositide turnover. *Life Sci.* 38:663-669
- Connors, M. and Rosencratz, H. (1962) Serotonin uptake and action in the adrenal cortex. *Endocrinology* 71:407-413.
- Corrent, G., McAdoo, D.J. and Eskin, A. (1978) Serotonin shifts the phase of the circadian rhythm from the Aplysia eye. *Science* 202 :977-979
- de Courcelles de Chaffoy de Didier, Leysen, J.E., de Clerck, F., Belle, H.V. and Janssen, P.A.J. (1985) Evidence that phospholipid turnover is the signal transducing system coupled to serotonin-S₂ receptor sites. *J. Biol. Chem.* 260(12):7603-7608
- Coughlin, S.R., Maskowitz, M.A., Levine, L. (1984) Identification of a type 2 receptor linked to prostacyclin synthesis in vascular smooth muscle cells. *Biochem. Pharmacol.* 33:692-695
- Coupland, R.E. (1965) The natural history of the chromaffin cell. Longmans Green
- Crawford, N. (1963) Plasma free serotonin (5-hydroxytryptamine). *Clin Chim. Acta* 8:39-45.

Creese, I., Burt, D.R. and Snyder, S.H. (1976) The dopamine receptor:differential binding of d-LSD and related agents to agonist and antagonist states. *Life Sci.* 17:1715-1720

Crickard, K., Ill, C.R. and Jaffe, R.B. (1981) Control of proliferation of human foetal adrenal cells in vitro. *J. Clin. Endocrinol. Metab.* 53(4):790-796

Crivello, J.F., Hornsby, P.J. and Gill, G.N. (1983) Suppression of cultured bovine adrenocortical zona glomerulosa aldosterone biosynthesis by steroids and its prevention by antioxidants. *Endocrinology* 113:235-242.

Cushman, P., Alter, S. and Hilton, J.G. (1966) Cortisol secretion in the dog adrenal : effects of cyclic adenosine monophosphate, dichloroisoproterenol, dihydroergotamine and adrenaline. *J. Endocrinol.* 34:271-272

Dallman, M. T., Engeland, W.C. and McBride, M.H. (1977) The neural regulation of compensatory adrenal growth in ACTH and related peptides: Structure, Regulation and Action(Krieger, D.T. and Ganong, W.F. eds) *Ann.N.Y.Acad.Sci USA* 297:373-392.

Davis, R.B. (1959) The concentration of serotonin in normal human serum as determined by an improved method. *J. Lab. Clin. Med.* 54:344-351.

Davis, J.O., Urquhart, J. and Higgins, J. (1963) The effects of alteration of sodium and potassium concentration on aldosterone production. *J. Clin. Invest.* 42:597-609

Davis, W.W., Burwell, L.R., Casper, A.G.T. and Bartter, F.C. (1968) Sites of action of sodium depletion on aldosterone biosynthesis in the dog. *J. Clin. Invest.* 47:1425-1434

Dawson-Hughes, B.F., Moore, T.J., Dluhy, R.G., Hollenberg, N.K. and Williams, G.H. (1981) Plasma AII concentration regulates vascular but not adrenal responses to sodium restriction in normal man. *Clin. Sci.* 61:527-534

Deane, H.W. and Greep, R.O. (1946) A morphological and histochemical study of the rats adrenal cortex after hypophsectomy with comments on the liver. *Am. J. Anat.* 79:114-145

Deane, H.W., Shaw, J.H. and Greep, R.O. (1948) The effect of altered sodium and potassium intake on the width and cytochemistry of the zona glomerulosa of the rat adrenal cortex. *Endocrinology* 43:133-153

De Lean, A., Gutkowska, J., McNicoll, N., Schiller, P.W., Canten, M. and Genest, G.(1984a) Characterisation of specific receptors for ANF in bovine adrenal zona glomerulosa . *Life Sci.* 35:2311-2318

De Lean, A. and Racz, K. (1984b) Interactions between adrenal zona glomerulosa and medulla enable inhibitory effects of catecholamines on aldosterone secretion in primary co-culture of bovine adrenal cells. Abstract 303 VII International Endocrine Meeting, Quebec

Dempster, I. (1978) Renal adrenal interrelationships Jpn. Ht. J. 19:426-433

Devynck, M., Pernollet, M., Matthews, P.G., Khosla, M.C., Bumpus, F.M. and Meyer, P. (1977) Specific receptors for [des-Asp¹-angiotensins II] ('AII') in rat adrenals. Proc. Natl Acad. Sci. 74:4029-4032.

Dihuy, R.G., Axelrod, L., Underwood, R.H. and Williams, G.H. (1972) Studies on the control of plasma aldosterone concentration in normal man. J. Clin. Invest. 51:1950-1957

Doi, Y., Atarachi, K., Franco-Saenz, R. and Mulrow, P.J. (1984) Effect of changes in sodium or potassium balance and nephrectomy, on adrenal renin and aldosterone concentrations. Hypertension 6:1124-129

Douglas J., Aguilera G., Kondo T. and Catt K.J. (1976a) Angiotensin II receptors and aldosterone production in rat adrenal glomerulosa cells. Endocrinology 102(3) :685-696.

Douglas J. and Catt K.J. (1976b) Regulation of AII receptors in the rat adrenal cortex by dietary electrolytes. J. Clin. Invest. 58:834-842.

Douglas, J., Aguilera, G., Kondo, T. and Catt, K.J. (1978a) Angiotensin II receptors and aldosterone production in rat adrenal glomerulosa cells. Endocrinology 102:685-696.

Douglas, J.G., Hansen, J. and Catt, K.J. (1978b) Relationship between plasma renin activity and plasma aldosterone in the rat after dietary electrolyte changes. Endocrinology 103:60-65.

Douglas, J. G., Michailov, M., Khosla, M.C. and Bumpus, F.M. (1979) Comparative studies of receptors binding and steroidogenic properties of AII in the rat adrenal glomerulosa. Endocrinology 104(1):71-75

Douglas, J.G (1980) Effects of high potassium diet on angiotensin II receptors and angiotensin - induced aldosterone production in rat adrenal zona glomerulosa cells. Endocrinology 106:983-900

Drake, C.R. and Carey, R.M. (1984) Dopamine modulates sodium dependent aldosterone responses to AII in humans. Hypertension 6(2):I119-I123

Dufau, M.L. and Kliman, B. (1968) Pharmacological effects of AII-amide in aldosterone and corticosterone secretion by the intact anaesthetised rat. Endocrinology 82:29-36

Dunn, G.M., Bruce H. and Bosmann, T. (1981) Peripheral dopamine receptor identification: Properties of a specific dopamine receptor in the rat adrenal zona glomerulosa. *Biochem. Biophys. Res. Commun.* 99(4):1081-1087

Edwards C.R.W., Thorney M.O., Miall P.A., Al Dujaili E.A.S., (1975) Inhibition of plasma aldosterone response to frusemide by bromocriptine. *Lancet* ii:903-905.

Edwards, C.R.W., Al-Dujaili, E.A.S., Boscaro M., Quyyumi, S., Miall, P.A. and Rees, L.H. (1980a) In vivo and in vitro studies on the effect of metoclopramide on aldosterone secretion. *Clin. Endocrinol.* 13:45-50

Edwards. C.R.W., Boscaro, M. Miall, P.A., Delitala, G. and Al-Dujaili, E.A.S. (1980b) Inhibition of aldosterone secretion by histamine-H₂ receptor antagonist. *Proceedings of VI International Cong of Endocrinol*:505 Abstr 592 (Melbourne, Australia)

Elliott, M.E. and Goodfriend, T.L. (1981) Angiotensin II alters ⁴⁵Ca²⁺ fluxes in bovine adrenal glomerulosa cells. *Proc. Natl. Acad. Sci USA* 78:3044-3048

Ellis, E.F., Shen, J.C., Schrey, M.P., Carchman, R.A. and Rubin, R.P. (1978) Prostacylin: A potent stimulator of adrenal steroidogenesis. *Prostaglandins* 16(4):483-490

Engbaek, F. and Voldby, B. (1982) Radioimmunoassay of serotonin in the cerebral spinal fluid, plasma and serum. *Clin. Chem.* 28 (4 Pt 1):642-648.

Engeland, W.C., Freeley, S. and Wilkie, C. (1982) Cholinergic reinnervation of adrenals transplanted to the anterior chamber. *Fed. Proc.* 41:1111 Abstr. 4919

Engeland, W.C., Lilly, M.P., Gann, D.S. (1985) Sympathetic adrenal denervation decreases adrenal blood flow without altering the cortisol response to haemorrhage. *Endocrinology* 117(3):1000-1010

Enjalbert, A., Bourgoin, S., Hamon, M., Adrien, J. and Bockaert, J. (1978) Postsynaptic 5HT sensitive adenylate cyclase in the central nervous system. *Mol. Pharmacol.* 14:2-10

Ersparmer, V. and Asero, J. (1952) Identification of enteramine, the specific hormone of the enterochromaffin cell system as 5-hydroxytryptamine. *Nature (London)* 169:800-801.

Erwin, V. G. and Deitrich, R.A. (1978) Brain aldehyde dehydrogenase : Localisation, purification and properties. *J. Biol. Chem.* 241: 3533-3539

Fakunding, J.L., Chow, R. and Catt, K.J. (1979):The role of calcium in the stimulation of aldosterone by ACTH, AII and potassium in isolated glomerulosa cells. *Endocrinology* 105(2):327-33

Farese, R.V., Sabir, M.A. and Larson, L.E. (1981) A23187 inhibits adrenal protein synthesis and the effects of adrenocorticotropin (ACTH) on steroidogenesis and phospholipid metabolism in rat adrenal cells in vitro : further evidence implicating phospholipids in the steroidogenic action of ACTH. *Endocrinology* 108(4):1243-1246

Farese, R.V., Larson, R.E., Sabir, M.A. and Gomez-Sanchez C.E.(1983) The effects of AII, potassium, ACTH, 5HT, adenosine 3, 5-monophosphate, guanosine 3, 5, monophosphate, A23187 and EGTA on aldosterone synthesis and phospholipid metabolism in the rat adrenal zona glomerulosa. *Endocrinology* 113(4):1377-1386

Fattah, I.D., Whitehouse, B.J. and Vinson, G.P. (1975) Synthesis of 18 hydroxysteroids in the rat adrenal cortex *J. Endocrinol.* 65:50P-51P

Fattah, D.I., Whitehouse, B.J. and Vinson, G.P. (1976) Effect of stimulation on biosynthesis of aldosterone from the late pathway precursors in the rat. *J. Endocrinol.* 69:32P-33P

Fattah, D.I., Whitehouse, B.J. and Vinson, G.P. (1977) Biosynthesis of aldosterone from 18 hydroxylated precursors by rat adrenal tissue in vitro *J. Endocrinol.* 75:187-195

Felder, R.A., Blecher, M., Eisner, G.M. and Jose, P.A. (1984) Cortical tubular and glomerular dopamine receptors in the rat kidney. *Am. J. Physiol.* 246:F557-F568

Few, J.D., Gawell, M.J., Imms, F.J. and Tiptaft, E.M. (1980) The influence of the infusion of norepinephrine on plasma cortisol levels in man. *J. Physiol.* 309:375-389

Fillion, G., Rouselle, J., Fillion, M., Beaudoin, D.M., Goiny, M.R., Deniau, J.M. and Jacob, J.J. (1978) High affinity binding of 3H 5HT to brain synaptosomal membranes: comparison with ³H LSD binding. *Mol. Pharmacol.* 14:50-59

Flack, J.D., Jessip, R. and Ramwell, P.W.(1969) Prostaglandin stimulation of rat corticosteroidogenesis. *Science* 163:691-692

Ford, J.K. and Young, R.W. (1963) Cell proliferation and displacement in the adrenal cortex of young rats injected with [³H]-thymidine. *Anat. Rec.* 146:125-138

Fozard, J.R. (1983a) Functional correlates of 5HT₁ recognition sites. *Trends Pharmacol. Sci.* 7(4):288-289

Fozard, J.R. (1983b) Mechanism of the hypotensive effect of ketanserin. J. Cardiovasc. Pharmacol. 4(5):829-38

Fraser, R., Brown, J.J., Lever, A.F., Mason, P.A. and Robertson, J.J.S. (1979) Control of aldosterone secretion. Clin. Sci. 56:389-399

Fraser, R., Berretta-Picoli, C., Brown, J.J., Cumming, A., Lever, A., Mason, P.A. et al., (1981) Responses of aldosterone and 18 hydroxycorticosterone to AII in normal subjects and patients with essential hypertension, Conns syndrome and non-tumorous hyperaldosteronism. Hypertension 3(PtII):187-192

Fredlund, P., Saltman, S. and Catt, K.J. (1975) Aldosterone production by isolated adrenal glomerulosa cells: stimulation by physiological concentrations of AII. Endocrinology 97:1577-1586.

Fredlund, P., Saltman, S., Kondo, T., Douglas, J. and Catt, K.J. (1977) Aldosterone production by isolated glomerulosa cells: modification of sensitivity to AII and ACTH by extracellular potassium concentration. Endocrinology 100:481-486.

Frisina, N., Buemi, M., Squadrito F. and Macri, I. (1986) Alpha- human natriuretic atrial peptide and aldosterone output: study in vitro Abstract 14. Serona Meeting, (Germany)

Fujita, K., Aguilera, G. and Catt, K.J. (1979) The role of cAMP in aldosterone production by isolated zona glomerulosa cells. J. Biol. Chem. 254(17):8567-8574

Fukuda, S., Su, C. and Lee, T.J.F. (1986) Mechanisms of extra-neuronal 5HT uptake in the rat aorta. J. Pharmacol. Exp. Ther. 239(1):264-269

Fuller, R.W. and Snoddy, H.D. (1980) Effect of 5HT releasing drugs on plasma corticosterone levels in rats. Neuroendocrinology 31:96-100

Fuller, R.W. (1981) Serotonergic stimulation of pituitary-adrenocortical function in rats. Neuroendocrinology 32:118-127

Fuxe, K., Butcher, L. and Engel, J. (1971) DL-5-hydroxytryptophan induced changes in central monoamine neurons after peripheral decarboxylase inhibition. J. Pharm. Pharmacol. 23:420-424

Ganguly, A. and Hampton, T. (1985) Calcium-dependence of serotonin-mediated aldosterone secretion and differential effects of calcium antagonists. Life Sci. 36:1459-1464

Ganong, W.F., Boryczka, A.T., Shackelford, R. Clark, R.M. and Converse, R.P. (1965) Effect dietary sodium restriction on adrenal cortical response to ACTH. Proc. Soc. Exp. Biol. Med. 118:792-795

Garcia, R., Cantin, M., Thibault, G., Ong, H. and Genest, J. (1982) Relationship of specific granules to the natriuretic and diuretic activity of rat atria. *Experimentia* 38:1071-1073.

Geaney, D.P., Schachter, W. and De Clerck, F. (1983) Characterisation of [³H]-LSD binding sites to a serotonin receptor on human platelets. *Eur. J. Pharmacol.* 97:87-93

Genefke, I.K. and Madel, P. (1968) Variations diurnes de la serotonine libre chez des sujets Normaux. *Clin. Chim. Acta* 19:131-138.

Gershon, M.D. and Ross, L.L. (1966) Radioisotopic studies of the binding, exchange, and distribution of 5HT synthesised from its radioactive precursor. *J. Physiol.* 186:451-476

Giguere, V., Cote, J. and Labrie, F. (1982) Specific inhibition by glucocorticoids of the α -1-adrenergic stimulation of adrenocorticotropin release in rat anterior pituitary cells. *Endocrinology* 110:1225-1230

Gilbert, J.C. and Goldberg, L.I. (1975) Characterisation by cyproheptadine of the dopamine induced contractions in canine isolated arteries. *J. Pharmacol. Exp. Ther.* 193(2):435-442.

Gill, G.N., Crivello, J.F., Hornsby, P.J. and Simonian, M.H. (1982) Growth, function and development of the adrenal cortex: insights from cell culture. *Cold Spring Harbor Conference on Cell Proliferation* 9:461-482.

Giroud, C.J.P., Stachenko, J. and Verning, E.H. (1956) Secretion of aldosterone by the zona glomerulosa cells: stimulation of the rat adrenal glands incubated in vitro. *Proc. Soc. Exp. Bio. Med.* 92:154-158

Gniadek, T.C., Grekin, R.J., Gross, M.D. and Villareal, J.Z. (1982) Hyper-responsiveness of aldosterone to metoclopramide in aldosteronism. *Clin. Endocrinol.* 16:475-481

Goodfriend, T.L. and Peach, M.J. (1975) Angiotensin II: (Des-aspartic acid¹)-angiotensin II. Evidence and speculation for its role as an important agonist in the renin-angiotensin system, *Circ. Res* 36 and 37 (Suppl. 1):1-38.

Gordon, M.B., Moore, T.J., Dluhy, R.G. and Williams, G.H. (1983) Dopaminergic modulation of aldosterone responsiveness to AII with changes in sodium intake. *J. Clin. Endocrinol. Metab.* 56(2):340-345

Gospodarowicz, D. and Handley, H.H. (1976) Stimulation of division of Y1 adrenal cells by growth factor isolated from bovine pituitary glands. *Endocrinology* 97:102-107

- Gow, I. F., Corrie, J.E.T., and Williams, B.C. and Edwards, C.R.W. (1987) Development and validation of an improved radioimmunoassay for serotonin in platelet-rich plasma. Clin. Chim. Acta 162:175-188
- Grahame-Smith, D.G., Butcher, R.W., Ney, R.L. and Sutherland, E.W. (1967) Adenosine 3', 5'-monophosphate as the intracellular mediator of the action of adrenocorticotrophin hormone in the adrenal cortex. J. Biol. Chem. 242:5535-5541.
- Green, J.P. and Furano, A.V. (1962) Two pools for amines in neoplastic mast cells. Biochem. Pharmacol. 11:1049-1053
- Green, A.R., Koslow, S.H. and Costa, E. (1973) Identification and quantitation of a new indoleethylamine in the rat hypothalamus. Brain Res. 51:371-372
- Greengard, P., Psychoyos, S., Tallan, H.H., Cooper, D.Y., Rosenthal, O. and Estabrook, R.W. (1967) Aldosterone biosynthesis by adrenal mitochondria III Participation of cytochrome P450. Arch. Biochem. Biophys. 121:298-303
- Grekin, R.J., Dale S.C. and Melby, J.C. (1973) The role of 18 hydroxydeoxycorticosterone as a precursor in human adrenal tissue in vitro. J. Clin. Endocrinol. Metab. 37:261-264
- Griffith, S.G. and Burnstock, G. (1983) Immunohistochemical demonstration of serotonin in nerves supplying human cerebral and mesenteric blood vessels. Lancet ii 12:561-562
- Griffing, G.T., Berelowitz, B., Hudson, M., Salzman, R., Manson, J., Aurrechia, S. and Melby, J.C. (1985) Plasma immunoreactive α -melanotropin in patients with idiopathic hyperaldosteronism, aldosterone producing adenomas, and essential hypertension. J. Clin. Invest. 76(1):163-169
- Gross, M.D., Grekin, R.J., Gniadek, T.C. and Villareal, J.Z. (1981) Suppression of aldosterone by cyproheptadine in idiopathic aldosteronism. N. Engl. J. Med. 305:181-185
- Guicheney, P., Baudoin-Legros, M., Garnier, J.P., Roques, P., Dreux, C., Meyer, P. (1985) Platelet serotonin and blood tryptophan in spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats. J. Cardiovasc. Pharmacol. 7 Suppl 7:S15-17
- Gullner, H. G. and Gill, J.R. (1983) β -endorphin selectively stimulates aldosterone secretion in hypophysectomised-nephrectomised dogs. J. Clin. Invest. 71:124-128
- Hadjian, A.J., Ventre, R., Chambaz, E.M. (1982) Cholinergic muscarinic receptors in bovine adrenal cortex. Biochem. Biophys. Res. Comm. 98(4):892-900

Halkerston, I.D.K. (1975) Cyclic AMP and adrenocortical function. Adv. Cyc. Nucl. Res. 6:100

Hanington, R., Tait, S.A.S. and Tait, J.F. (1970) In vitro effects of ACTH, angiotensins, serotonin and potassium on steroid output and conversion of corticosterone to aldosterone by isolated adrenal cells. Endocrinology 87:1147-1167.

Hannah, J.A.M., Inglis, G.C., Connell, J.M., Davies, D.L., Fraser, R. and Ball, S.G. (1984) Dopamine, noradrenaline and adrenaline in the adrenal medulla and cortex of the rat Medical Research Society, Clin. Sci. 66:65P Abstr 189.

Harper, J.F. and Brooker, G. (1975) Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2'0 acetylation by acetic anhydride in aqueous solution. J. Cyclic Nucleotides Res. 1 (4):207-218.

Hata, T., Bravo, L., Sen, S. and Bumpus, M. (1984) Steroidogenic properties of a new aldosterone stimulating factor: interaction with AII and ACTH during variations in dietary sodium. Endocrinology 115(6):2065-2070

Hauger, R.L., Aguilera, G. and Catt, K.J. (1978) Angiotensin II regulates its receptor sites in the adrenal glomerulosa zone. Nature 271:176-178

Hay, D.W. and Wadsworth, R.M. (1982) Effects of some organic calcium antagonists and other procedures affecting calcium on KCl-induced contractions in the rat vas deferens. Br. J. Pharmacol. 76(1):103-113

Hervonean, A., Pelto-Huikko, M. and Linnoila, I. (1980) Ultrastructural localisation of enkephalin-like immunoreactivity in the rat adrenal medulla. Am. J. Anat. 157:445-448.

Hirata, Y., Uchihashi, M., Sueoka, S. Matsouka, S. and Fujita, T. (1981) Presence of ectopic beta-adrenergic receptors on human adrenocortical cortisol-producing adenomas. J. Clin. Endocrinol. Metab. 53 (5):953-957.

Hokfelt, T., Elvin, L-G., Schultzbergh, M., Fahrenkrug, J. (1981) Immunohistochemical evidence for a local VIP neurone system in the adrenal gland of the rat. Acta Physiol. Scand 113:575-576.

Hollenberg, N.K., Chenitz, W.R., Adams, D.F. et al., (1974) Reciprocal influence of salt intake on adrenal glomerulosa and renal vascular responses to AII in normal man. J. Clin. Invest. 54:34-42.

Holzwarth, M.A., Shinsako, J. and Dallman, M.F. (1980) Adrenal regeneration, Time course, effects of hypothalamic hemi-islands and response to unilateral adrenalectomy. Neuroendocrinology 31:168-176

- Holzwarth, M.A., Brownfield, M.S. (1983) Serotonin immunoreactivity in the adrenal medulla of the rat. Soc. Neurosc. Abstract 9:388
- Holzwarth, M.A. (1984) The distribution of VIP in the adrenal cortex and medulla. J. Anat. Nerv. Syst. 11:269-283
- Hornsby, P.J., O'Hare, M.J. and Neville, A.M. (1974) Functional and morphological observations on rat zona glomerulosa cells in monolayer culture. Endocrinology 95:1240-1251
- Hornsby, P.J. and Gill, G.N. (1977) Hormonal control of adrenocortical cell proliferation. J. Clin. Invest. 60:342-352
- Hornsby, P.J. (1982) Regulation of 21-hydroxylase activity by steroids in cultured bovine adrenocortical cells: possible significance for adrenocortical androgen synthesis. Endocrinology 111(4):1092-1101
- Hornsby, P.J. and Crivello, J.F. (1983) The role of lipid peroxidation in biological antioxidant in the function of the adrenal cortex. Part 2 Mol. Cell. Endocrinol. 30:123-147
- Hornsby, P.J. and Alderm, K.A. (1984) Steroidogenic Enzyme activities in cultured human definitive zone adrenocortical cells: Comparisons with bovine adrenocortical cells and resultant differences in adrenal androgen synthesis. J. Clin. Endocrinol. Metab. 58:121-130
- Hornsby, P.J. (1985) 'The Regulation of Adrenocortical Function by Control of Growth and Structure' in Adrenal Cortex, (pp1-31) (Anderson, D.C. and Winter, J.S.D. eds), Butterworths
- Hoyer, D., Engel, G. and Kalkman, H.O. (1985) Molecular pharmacology of 5HT₁ and 5HT₂ recognition sites in rat and pig brain membranes: radioligand binding sites with [³H]-5HT, [³H]-8OH-DPAT, (-)[¹²⁵I]-Iodocyanopindolol, [³H]-mesulergine and [³H]-ketanserin. Eur. J. Pharmacol. 118:13-23
- Humphrey, J.H. and Toh, C.C. (1954) Absorption of 5 hydroxytryptamine (5HT) and histamine by dog platelets. J. Physiol. 124:300-304.
- Idelman, S. (1978) 'The Structure of the Mammalian Adrenal Cortex' in General, Comparative and Clinical Endocrinology (Vol. 2), (Jones, I.C. and Henderson, I.W eds), Academic Press
- Janowsky, A., Okada, F., Manier, D.H., Applegate, C.D. and Salser, F. (1982) Role of serotonergic input in the regulation of the beta adrenergic receptor-coupled adenylate cyclase system. Science 218:900-901

Jefcoate, C.R., Simpson, E.R. and Boyd, G.S. (1974) Spectral properties of rat adrenal mitochondrial cytochrome P450. Eur. J. Biochem 42:539-551

Jones, M.T. and Hillhouse E.W. (1977) Neurotransmitter regulation of corticotropin releasing factor in vitro. Ann N Y Acad Sci 292: 536-560

Jones, D.B., Marante, D., Williams, B.C. and Edwards, C.R.W. (1986) Adrenal synthesis of corticosterone in response to ACTH in rats is influenced by leukotriene A₄ and by lipoxygenase intermediates. J. Endocrinol. 112:253-258.

Johnson, B.B., Lieberman, A.H. and Mulrow (1957) Aldosterone excretion in normal subjects depleted of sodium and potassium. J. Clin. Invest. 36:757-766

Kamal, L.A., Le Quan-Bui, K.H. and Meyer, P. (1984) Decreased uptake of [³H]-5HT and endogenous content of 5HT in blood platelets in hypertensive patients. Hypertension 6:568-570.

Kaplan, N.M. (1965) The biosynthesis of adrenal steroids; effects of angiotensin II, ACTH and potassium. J.Clin. Invest. 44:2029-2039

Kellum, J.M. and Jaffe, B.M. (1976) Validation and application of a radioimmunoassay for serotonin. Gastroenterology 70:516

Kinson, G.A. and Singer, B. (1968) Sensitivity to AII and ACTH in the sodium deficient rat. Endocrinology 83:1108-1116.

Kiss, V.T. (1951) Experimentell- morphologische analyse der nebennierenninnervation. Acta Anat. 13:81-89

Klietman, N. and Holzwarth, M.A. (1985) Catecholaminergic innervation of the rat adrenal cortex. Cell Tiss. Res. 241:139-147.

Koe, B.K. and Weissman, A. (1966) para-Chlorophenylalanine (PCPA): a specific depletor of brain 5HT. J. Pharmacol. Exp. Ther. 154:499-516.

Kojima, I., Kojima, K., Kreutter, D. and Rasmussen, H. (1984) The temporal integration of the aldosterone secretory response to AII occurs via two intracellular pathways. J. Biol. Chem. 259:14448-14457.

Kojima, I., Kojima, K. and Rasmussen, H. (1985) Intracellular calcium and adenosine 3',5'-cyclic monophosphate as mediators of potassium-induced aldosterone secretion. Biochem J. 228:69-76.

Koletsky, R.J., Brown, E.M. and Williams, G.H. (1983) Calmodulin-like activity and calcium-dependent phosphodiesterase in purified cells of the rat zona glomerulosa and zona fasciculata. Endocrinology 113:485-490.

Kolta, M.G. and Soliman, K.F.A.(1981) Effect of peripheral cholinergic activation on the adrenal cortex function. *Endo. Res. Commun.* 8(4):239-246.

Kono, T., Oseko, F., Shimpo, S., Nanno, M. and Endo, J.(1975) Biological activity of des-Asp-angiotensin II (AIII) in man. *J.Clin Endocrinol. Metab.* 41:1174-1177.

Kramer, R.E., Gallant, S. and Brownie, A.C. (1979) The role of cytochrome P450 in the action of sodium depletion on aldosterone biosynthesis in rats. *J.Biol. Chem.* 254:3953-3958.

Kramer, R.E., Gallart, S. and Brownie, A.C. (1980) Actions of AII on aldosterone biosynthesis in the rat adrenal cortex. Effects on cytochrome P450 enzymes of the early and late pathway. *J. Biol. Chem.* 255(8):3442-3447.

Krieger, D.T., Krieger, H.P. (1970) Chemical stimulation of the brain and the effects on adrenal corticosteroid release. *Am. J. Physiol.* 218:1632-1641.

Krieger, D.T.(1977) Serotonin regulation of ACTH secretion. *Ann. N. Y. Acad. Sci.* 297:527-534.

Krulich, L., Coppins, R.J., McCaan and Mayfield, M.A.S. (1978) Inhibition of prolactin secretion by a direct effect of methysergide on the pituitary lactotrophs in the rat. *Life Sci.* 23:1665-1673.

Krulich, L., Coppins, R.J., McCaan, S.N. (1981) On the mode of prolactin release-inhibiting action of the serotonin receptor blockers, metergoline, methysergide and cyproheptadine. *Endocrinology* 108:1115-1124.

Kudlow, J.E. and Genie, B.M. (1983) Production of growth factor activity by cultured bovine calf anterior pituitary cells. *Endocrinology* 113:104-110.

Kuriyama, K., Kanmori, K. and Yoneda, Y. (1984) Preventive effect of alcohol against stress-induced alteration in content of monoamines in brain and adrenal gland. *Neuropharmacology* 23 (6):649-654.

Kuramoto, H., Kondo, H. and Fijita, T. (1985) Substance P like immunoreactivity in adrenal chromaffin cells and intra-adrenal nerve fibres in the rat. *Histochemistry* 82(6):507-512.

Lackovic, Z., Relja, M. and Neff, N.H. (1982) Catabolism of endogenous dopamine in peripheral tissues: is there a role for dopamine in peripheral neurotransmission. *J. Neurochem.* 38:1453

Lamberts, S.J.W. and Macleod, R. (1978) The interaction of the serotonergic and dopaminergic systems on prolactin secretion in the rat. *Endocrinology* 103:287-294.

Landsteiner, K. (1947) The specificity of serological reactions. Howard University Press, Cambridge.

Lauer, C.G., Braley, L.M., Menarchery, A.I. and Williams, G.H. (1982) Metoclopramide inhibits aldosterone biosynthesis in vitro. Endocrinology 111:238-243.

Laychock, S.G., Waines, W and Rubin, R.P. (1977) Further studies on the mechanism controlling prostaglandin biosynthesis in the cat adrenal cortex. Endocrinology 100:74-81.

Lee, T.C., Van Der Waal, B., De Wied, D. (1968) Influence of the anterior pituitary on the aldosterone secretory response to dietary restriction in the rat. J. Endocrinol. 42:465-475.

Leutscher, J.A. and Axelrad, B.J. (1954) Increased aldosterone output during sodium deprivation in man. Proc. Soc. Exp. Biol. (NY) 87:650-653.

Lever, J.D. (1953) Nerve fibres in the adrenal cortex of the rat. Nature 171:882-883.

Leysen, J.E., Awouters, F., Kennis, L., Laduron, P.M., Van der bak, J., and Janssen, P.A. (1981) Receptor binding profile of R 41 468, a novel antagonist at 5HT 2 receptors. Life Sci. 28:1015-1022.

Leysen, J.E., Niemegeers, C.J., Van Neuten, J.M., and Laduron, P.M. (1982) [³H]-ketanserine (R 41 468) a selective ³H-ligand for serotonin 2 receptor binding sites. Binding properties, brain distribution and functional role. Mol. Pharmacol. 21:301-304.

Leysen, J. (1983) Serotonin receptor sites: Is there pharmacological and clinical existence? Med. Biol. 61:139-143.

Lieberman, A.H. and Luetscher, J.A. Jr (1960) Some effects of abnormalities of pituitary, adrenal or thyroid function on excretion of aldosterone and the response to corticotropin or sodium deprivation. J. Clin. Endocrinol. 20:1004-1016.

Linnoila, R.I., DiAugustine, R.P. and Hervouen, A. (1980) Distribution of [Met⁵]- and [Leu]-enkephalin, VIP and substance P like immunoreactivity in human adrenal glands. Neuroscience 5:2247-2259.

Lis, M., Hammet, P., Gutkowska, J., Maurice, G., Seidah, N.G., Lariviere, N. et al., (1981) Effect of N-terminal portion of pro-opiomelanocortin on aldosterone release by human adenoma in vitro. J. Clin. Endocrinol. Metab. 52:1053-1056.

Livett, R.G. (1984) Adrenal medullary chromaffin cells in vitro. Physiol Rev 64(4):1103-1156.

Lowry, P.J., Silas, L., McLean, C., Linton, E.A. and Estivariz, F.E. (1983) Pro-gamma-melanocyte stimulation hormone cleavage in adrenal gland undergoing compensatory growth. *Nature* 306:70-73.

Luborsky-Moor, J.L. and Jacklet, J.W. (1976) Localisation of catecholamines in the eye and other tissues of Aplysia. *J. Histochem. Cytochem* 24:1150-1158.

Lun, S., Espiner, E.A., Nicholls, G.M. and Hart, D.S. (1983) Lack of direct effect of dopamine on aldosterone secretion in vivo. *Endocrinology* 112(1):60-63.

Lundquist, I., Hakanson, R., Sundler, F. and Heding, L.G. (1978) Serotonin and insulin co-stored in guinea pig beta cells: 5HT and insulin storage mechanisms. *Diabetologia* 15:251 Abstr 214.

McCaa, R.E., Young, D.B., Guyton, A. G. and McCaa, C.S. (1974) Evidence for a role of unidentified pituitary factor in regulating aldosterone secretion during altered sodium balance. *Circ Res. (Suppl 1)* vols 43 and 35 :1-15.

McCaa, R.E., Montalvo, J. and McCaa, C.S. (1978) Role of growth hormone in the regulation of aldosterone biosynthesis. *J. Clin. Endocrinol.Metab.* 46:247-253.

McCarty, R., Kirby, R.F. and Carey, R.M. (1984) Dopamine may be a neurohormone in rat adrenal cortex. *Am J. Physiol.* 247:E709- E 713.

McKenna, J.T., Island, D.P. Nicholson, W.E. and Liddle, G.W. (1978a) The effects of potassium on the early and late steps in aldosterone biosynthesis in cells of the zona glomerulosa. *Endocrinology* 103(4):1411-1416.

McKenna, J.T., Island, D.P., Nicholson, W.E. and Liddle, G.W. (1978b) AII stimulates the early and late steps in aldosterone biosynthesis in isolated bovine zona glomerulosa cells. *J. Steroid Biochem.* 9:967-972.

McKenna, J.T., Island, D.P., Nicholson, W.E. and Liddle, G.W. (1979) Dopamine inhibits AII stimulated steroidogenesis in bovine adrenal cells. *J. Clin. Invest.* 64:287-291.

Mackie, C., Richardson, M.C. and Schulster, D. (1972) Kinetics and dose response characteristics of adenosine 3', 5'-monophosphate production by isolated rat adrenal cells stimulated with adrenocorticotrophic hormone. *FEBS Lett* 23(3):345-348.

Mackie, C.M., Simpson, E.R., Mee, M.S., Tait, S.A. and Tait, J.F. (1977) Intracellular potassium and steroidogenesis of isolated rat adrenal cells : effects of potassium ion and AII on purified zona glomerulosa cells. *Clin. Sci. Mol. Med* 53 (3):289-296.

Mackie, C., Warren, R.L. and Simpson, E.R. (1978) Investigation into the role of calcium in the control of steroid production by isolated adrenal zona glomerulosa cells of the rat. *J. Endocrinol.* 77(1):119-127.

Maestri, E., Camellini, L., Montanari, R., Rossi, G. and Gnudi, A. (1985) Aldosterone regulation: A role for serotonin? in *The Adrenal Gland and Hypertension* Vol. 27, pp 97-100, (Mantero, F., Biglieri, E.G., Scoggins, B.A. and Funder, F.W. eds), Symposium Publications, Raven Press.

Mais, D.E., Lahr, P.D. and Bosin, T.R. (1981) Determination of serotonin, its precursors, metabolites and [³H]-5HT in the lung by HPLC with fluorescence detection. *J. Chromatogr.* 225 (1):27-35.

Maki, M., Takayariagi, R., Misono, K.S., Pandey, K.N., Tibbetts, C. and Inagami, T. (1984) Structure of the rat atrial factor precursor from cDNA 'sequence. *Nature* 309:722-724.

Mantero, F., Opocher, G., Armanini, D., Boscaro, M. and Edwards, C.R.W. (1979) Effect of serotonin (5HT) on plasma aldosterone in Man. *Acta Endocrin (Suppl.225):345*

Mantero, F., Rocco, S., Opocher, G., Armanini, D., Boscaro, M. and D'Agastino, D. (1985) Effect of ketanserin primary aldosteronism. *J. Cardiovasc. Pharmacol.* 7 (Suppl 17):S172-S175.

Marieb, N.J. and Mulrow, P.J. (1965) Role of the renin-angiotensin system in the control of aldosterone secretion in the rat. *Endocrinology* 76:579-585.

Marusic, E.T. and Mulrow, P.J. (1975) Stimulation of aldosterone biosynthesis in adrenal mitochondria in sodium depletion. *J. Clin. Invest.* 40:2101-2109.

Matsouka, H. and Mulrow, P.J. (1980) Beta lipotropin: a new stimulating factor. *Science* 209: 307-308.

Matsouka, H., Mulrow, P.J. and Franco-Saenz, R. (1981a) Effects of beta lipotropin derived peptides on aldosterone production in the rat adrenal gland. *J. Clin Invest.* 68:752-759.

Matsouka, H., Mulrow, P.J., Franco-Saenz and Li, C.H. (1981b) Stimulation of aldosterone production by beta melanotropin. *Nature* 291:155-156.

Mayer, G.G. and Shoup, R.E. (1983) Simultaneous multiple electrode liquid chromatographic-electrochemical assay for catecholamines, indoleamines and metabolites in brain tissue. *J. Chromatogr.* 255:533-544.

Mazzochi, G., Rebuffat, P. and Robba, C.I. (1985) Trophic effects of potassium loading in the rat zona glomerulosa: permissive role of ACTH and AII. *Acta Endocrinol. (Copenh)* 108 (1):98-103.

Mefford, I.N. (1981) Application of HPLC with electrochemical detection to neurochemical analysis: measurement of catecholamines, serotonin and metabolites in the rat brain. *J. Neurosci. Meths.* 3:207-224.

Melander, A., Owman, C. and Sunder, F. (1971) TSH induced apperance and stimulation of amine containing mast cells in the mouse thyroid. *Endocrinology* 95:528-533.

Meltzer, H.Y., Wiita, B., Tricou, B.J. et al., (1982) Effects of 5HT precursors and 5HT agonists on plasma hormone levels in Serotonin in *Biological Psychiatry* (Ho, B. T. ed.), Raven Press.

Mendelsohn, F.A. and Mackie, C. (1975) Relation of intracellular potassium and steroidogenesis in isolated adrenal zona glomerulosa and fasciculata cells. *Clin Sci. Mol. Med.* 49:13-26.

Mendelsohnn, F.A. and Rachel, C.D. (1981) Stimulation by serum of aldosterone production by rat adrenal glomerulosa cells in vitro: relationship to potassium, 5HT and AII. *Acta Endocrinol. (Copenh)* 97 (2):231-242.

Messereli, F.H., Nowaczynski, W., Honda, M., Genest, G., Boucher, R., Kuchel, O. and Rojo-Ortega, J.M. (1977) Effects of AII on steroid metabolism and heaptic blood flow in Man. *Circ. Res.* 40:204-207

Messing, R.B., Fisher, L.A. and Phebus, L. (1976) Interaction of diet and drugs in the regulation of brain 5 hydroxyindoles and the response to painful electric shock. *Life Sci.* 18:707-714.

Meuli, C. and Muller, J. (1983) Potassium intake and aldosterone biosynthesis: the role of cytochrome P450. *J. Steroid Biochem.* 18:167-171.

Michell, R.H., Jones, L.M. and Jafferl, S.S. (1977) A possible role for phosphoinositol breakdown in muscarinic, cholinergic stimulus-response coupling. *Biochem. Soc. Trans* 5:77-81.

Migally, N. (1979) The innervation of the mouse adrenal cortex. *Anat. Rec.* 194:105-112.

Mikhail, Y. (1961) Innervation of the different zones of the dreanl cortex. *J. Comp. Neur.* 117: 365-369.

Mikhail, Y and Mahran, Z. (1965) Innervation of cortical and medullary portions of the adrenal gland of the rat during post-natal life. *Anat. Rec.* 152:431-438.

Miller, F.P. Cox, R.H. Jr. and Snodgrass, W.R. (1970) para-chlorophenylalanine, para-chlorophetamine and para-chloro-N-methylamphetamine on rat brain norepinephrine, serotonin and 5-hydroxyindole-3-acetic acid. *Biochem. Pharmacol.* 19:435-442.

Missale, C., Liberini, P., Memo, M., Caruba, M. and Spano, P.F. (1985) ^3H sulpiride binding in D2 receptors in bovine adrenal cortex. *Life Sci.* 37:2539-2548.

Missale, C., Liberini, P., Memo, M., Carruba, M.O. and Spano, P. (1986) Characterisation of dopamine receptors associated with aldosterone secretion in rat adrenal glomerulosa. *Endocrinology* 119 (5):2227-2232.

Modlinger, R.S., Schonmuller, J.M. and Arora, S.P. (1979) Stimulation of aldosterone, renin and cortisol by tryptophan. *J. Clin. Endocrinol. Metab.* 48(4):599-603.

Morise, T., Miyamori, I. and Takeda, R. (1982) Role of prostaglandins in the aldosterone response to ACTH in sodium depleted human subjects. *Clin. Exp. Hypertension- Theory and Practice* A4 (9 and 10):1469-1479.

Müller, J. (1965) Aldosterone stimulation in vitro I Evaluation of assay procedure and determination of aldosterone stimulating factor in human urine extract. *Acta Endocrinol.* 48:283-296.

Müller, J. (1966) Aldosterone stimulation in vitro III Site of action of different aldosterone stimulating substances in steroid biosynthesis. *Acta Endocrinol.* 52:515-526.

Müller, J. and Weick, W.J. (1967) Aldosterone stimulation in vitro. IV Corticosteroid-stimulating activity of rat serum. *Acta Endocrinol.* 54:63-72.

Müller, J. and Ziegler, W.J. (1968) Stimulation of aldosterone biosynthesis in vitro by serotonin. *Acta Endocrinol.* 59:23-35.

Müller, J. (1970) Steroidogenic effect of stimulators of aldosterone biosynthesis upon separate zones of the rat adrenal cortex. *Eur. J. Clin. Invest.* 1:180-187.

Muller, E.E., Panevai, A.E. Cocchi, D. and Mantegazza, P. (1977) Endocrine profile of ergot alkaloids. *Life Sci.* 21:1545-1558.

Muller, J. (1980) The conversion of 18OHB and 18OHDOC to aldosterone by rat adrenal tissue: evidence for alternative biosynthetic pathway. *J. Steroid Biochem.* 13:245-251.

Mulrow, P.J. and Ganong, W.F. (1961) The effect of haemorrhage upon aldosterone secretion in normal and hypophysectomised dogs. *J. Clin. Invest.* 40:579-585.

Nahmod, V.E. Finkielman, S., Benarroch, E.E. and Pirola, C.J. (1978) Angiotensin regulates release and synthesis of 5HT in brain. *Science* 202 (8):1091-1093.

Nakanishi, S., Inone, A., Kita, T., Nakamura, M., Chang, A.C. Y., Cohen, S.N. and Numa, S. (1979) Nucleotide sequence of cloned cDNA for bovine corticotropin- beta lipotropin precursor. *Nature* 278:423-427.

Nelson, D.L., Herbert, A., Bourgoin, G., Glowinski, J. and Hamon, M. (1978) Characteristics of central serotonergic 5HT receptors and their adaptive changes following intracerebral 5, 7, dihydroxytryptamine administration in the rat. *Mol. Pharmacol.* 14:983-995.

Nelson, D.L., Herbert, A., Enjalbert, A. Bockaert, J. and Hamon, M. (1980) Serotonin sensitive adenylate cyclase and ^3H serotonin binding sites in the CNS of the rat I. Kinetic parameters and pharmacological properties. *Biochem. Pharmacol.* 29:2445-2453.

Nicholson, M.D., Swann, R.W., Burford, G.D., Wathes, D.C., Porter, D.G. and Pickering, B.T. (1984) Identification of oxytocin and vasopressin in the testis and adrenal tissue. *Regulatory Peptides* 8:141-146.

Niemegeers, C.J.E., Colpaert, F.C., Leysen, J.E. et al., (1983) Mescaline-induced head twitches in the rat: an *in vivo* method to evaluate serotonin S₂ antagonists. *Drug Dev. Res.* 3:123-135.

Niijima, A. and Waiter, D.L. (1968) Baroreceptors in the adrenal gland. *Science* 159:434-435.

Niles, L.P., Brown, M. and Mishra, R.K. (1983) Characteristics of high affinity binding of ^3H N-Acetylserotonin in rat brain. *Neuropharmacology* 22(11):1311-1314.

Nilsson, O., Ericson, L.E., Dählström, A., Ekholm, R., Steinbusch, H.W.M. and Ahlman, H. (1985) Subcellular localisation of serotonin immunoreactivity in rat enterochromaffin cells. *Histochemistry* 82:351-356.

Nisbet, J. (1987) The role of serotonin (5HT) in the control of aldosterone secretion. Pharmacology Honours Project. Edinburgh University.

Norbiata, G., Berilacqua, M., Raggi, U., Micossi, P. and Moroni, C. (1977) Metoclopramide increases plasma aldosterone concentration in Man. *J. Clin. Endocrinol. Metab.* 45:1313-1316.

Noth, R.H., McCallum, R.W., Contino, C. and Havelick, J. (1980) Tonic dopaminergic suppression of plasma aldosterone. *J. Clin. Endocrinol. Metab.* 51(1):64-69.

Nussey, S.S., Ang, Y.V.T., Jenkins, J.S., Chowdrey, H.S. and Bisset, G.W. (1984) Brattleboro rat adrenal contains vasopressin. *Nature* 310:64-66.

Oelkers, W., Brown, J.J., Fraser, R., Lever, A.F., Morton, J.J. and Robertson, J.I.S. (1974) Sensitization of the adrenal cortex in sodium depletion in man. *Circ. Res.* 34:69-77.

Oelkers, W., Schonesshofer, M., Schultze, G. et al (1975) Effect of prolonged low dose AII infusion on the sensitivity of the adrenal cortex in man. *Circ Res.* 36 (6 Suppl 1):49-56

Ogle, T.F. and Kitray, J.J. (1978) In vitro effects of melatonin and 5HT on adrenal steroidogenesis. *Proc. Soc. Exp. Biol. Med.* 157:103-105.

*

Osim, E.E. and Wyllie, J.H. (1983) Loss of 5 hydroxytryptamine from mammalian circulating labelled platelets. *J. Physiol.* 340:77-90.

Osunkwo, U.A., Ngur, D. and Eferakeya, A. (1986) Plasma serotonin levels at different phases of experimental hypertension. *Physiology [IRCS] Med. Sci.* 14:998.

Ottenweller, J.E., Meuer, A.H. Ferrel, B.R. Horseman, N.D. and Proctor, A. (1978) Extrapituitary regulation of the circadian rhythm of plasma corticosteroid concentration in rats. *Endocrinology* 103:1875-1879.

Ottenweller, J.E. and Meier, A.H. (1982) Adrenal innervation may be an extrapituitary mechanism able to regulate adrenocortical rhythmicity in rats. *Endocrinology* 111:1334-1338.

Padfield, P., Davies, D., Lever, A., Brown, J.J., Fraser, R., Morton, J.J. and Robertson, J.I.S. (1981) The myth of idiopathic hyperaldosteronism. *Lancet* ii:83-84.

Page, I.M. (1958) Serotonin (5HT): the last four years. *Physiol. Rev.* 38:277-335.

Palmore, W.P. and Mulrow, P.J. (1967) Control of aldosterone secretion by the pituitary gland. *Science* 158:1482.

Palmore, W.P., Marieb, N.J. and Mulrow, P.J. (1969) Stimulation of aldosterone secretion by sodium depletion in nephrectomised rats. *Endocrinology* 84:1342-1350.

Palmore, W.P., Anderson, R. and Mulrow, P.J. (1970) Role of the pituitary in controlling aldosterone production. *Endocrinology* 86:728-734.

* Osborne, N (ed). (1982) 'The Biology of Serotonergic Transmission', John Wiley and Sons.

Parker, R.C., Carr, B.R., Winker, C.A., Casey, M.L., Simpson, E.R. and MacDonald, P.C. (1983) Hypercholesterolaemia due to elevated low density lipoprotein-cholesterol in newborns with anencephaly and adrenal atrophy. *J. Clin. Endo. Metab.* 57(1):37-43.

Payet, N. and Isler, H. (1976) Adrenal glomerulosa mitotic stimulation by posterior pituitary hormones. *Cell Tiss. Res.* 172:93-101.

Payet, N., Lehoux, J.G. and Isler, H. (1980) Effect of ACTH on proliferative and secretory activities of the adrenal glomerulosa. *Acta Endocrinol.* 93:365-374.

Payet, N. and Lehoux, J.G. (1982) Aldosterone and corticosterone stimulation by ACTH in isolated rat adrenal glomerulosa cells: Interaction with vasopressin. *J. Physiol. (Paris)* 78:317-321.

Payet, N., Deziel, Y. and Lehoux, J.G. (1984) Vasopressin a potent growth factor in adrenal zona glomerulosa cells in culture. *J. Steroid. Biochem.* 20:449-454.

Pederson R.C. and Brownie A.C. (1980a) Pro-adrenocorticotropin/endorphin-derived peptides: coordinate actions on adrenal steroidogenesis. *Science* 208:1044-1055.

Pederson R.C., and Brownie A.C. (1980b) Adrenocortical response to corticotrophin is potentiated by some part of the terminal region (16k) of pro-corticotropin/endorphin. *Proc Natl Acad Sci U.S.A.* 77:2239-2243.

Pederson R.C. and Brownie A.C. (1983) Cholesterol side chain cleavage in the rat adrenal cortex: isolation of a cycloheximide sensitive activator peptide. *Proc Natl Acad Sci U.S.A.* 80:1882-1886.

Pederson R.C., Teruel L., Alfano J.A. and Brownie, A.C. (1986) Mechanism of action of pro-gamma-MSH on adrenal steroidogenesis. Abstr 44 in Serona Symposium- Corticosteroids and Peptide Hormones in Hypertension Sept 6-7 Mannheim, Germany.

Pellmar T.C., Carpenter D.O. (1979) Voltage dependent calcium current induced by 5HT. *Nature* 277:483-484.

Peroutka S.J. and Snyder S.H. (1979) Multiple serotonin sites: Differential binding of [³H]-5HT, [³H]-LSD and [³H]-spiroperidol. *Mol Pharmacol* 16:687-699.

Peroutka S.J. and Snyder S.H. (1980) Long term antidepressant treatment decreases spiroperidol - labelled 5HT receptor binding. *Science* 210:88-90

Peroutka S.J., Noguchi M., Tolner D.J. and Allen, G.S. (1982) Serotonin-induced contractions of canine basilar artery: mediation by 5HT₁ receptors. Brain Res 259:327-330.

Peroutka, S.J. (1984) Vascular 5HT receptors. Correlation with 5HT₁ and 5HT₂ binding sites. Biochem. Pharmacol. 33 (15):2349-2353.

Petraglia F., Facchineti F., Mantignoni E., Nappi, G., Volpe, A. and Genazzani, A.R. (1984) Serotonergic agonists increase plasma levels of β -endorphin and beta lipotropin in humans. J. Clin. Endocrinol. Metab. 59(6):1138-1142.

Petrovic T (1984) Small fluorescent cells in the rat kidney contain 5-hydroxytryptamine not a catecholamine. J. Aut. Nerv. Sys. 11:329-338.

Podesta E.J., Milani A., Steffen and Neher, R. (1980) Steroidogenic action of calcium in isolated adrenal cortex. Biochem J. 186(2):391-397.

Ponzio, F. and Jonsson, G. (1979) A rapid and simple method for the detection of picogram levels of 5HT in brain tissue using liquid chromatography with electrochemical detection. J Neurochem 32:129-132.

Popova N.K., Maslova L.N. and Naumenko E.V. (1972) Serotonin and the regulation of the pituitary-adrenal axis after deafferentation of the hypothalamus. Brain Res. 47:61-67.

Potter, L.T. and Axelrod, J. (1963) Subcellular localisation of catecholamines in tissues of the rat. J. Pharmacol. Exp. Ther. 142:291-298.

Pratt J.H., Ganguly A., Parkinson C., and Weinberger, M.H. (1981) Stimulation of aldosterone secretion by MCP in humans: apparent independence of renal and pituitary mediation. Metabolism 30:129-134.

Pratt J H (1982) Role of AII in potassium mediated stimulation of aldosterone secretion in the dog. J Clin Invest 70(3):667-672.

Pratt J.H., Turner D.A., Mcateer J. and Henry, D.P. (1985) β -adrenergic stimulation of aldosterone production by rat adrenal capsular extracts. Endocrinology 117(3):1189-1194.

Racz K., Wolf I., Kiss R., Lada, G. and Glaz, E. (1979) Corticosteroidogenesis by isolated human adrenal cells: Effects of 5HT and 5HT antagonists Experientia 35:1532-1534.

Racz K., Buu N.T., Kuchel O. and De Lean, A. (1984) Dopamine 3-sulphate inhibits aldosterone secretion in cultured bovine adrenal cells Am J Physiol 247:E431-E435.

Rapport M.M., Green A.A. and Page I.H. (1948) Crystalline serotonin. Science 108:329-330.

Rasmussen, H. and Barrett, P.Q. (1984) Calcium messenger systems: An integrated view. Physiol. Rev. 64:938-984.

Robinson P.M., Perry R.A., Hardy K.J., Coughlin, J.P. and Scoggings, B.A. (1977) The innervation of the adrenal cortex in the sheep, Ovis ovis. J. Anat 124(1):117-129.

Rosenkrantz, H. and Laferte, R.O. (1960) Further observations on the relationship between 5HT and the adrenal. Endocrinology 67:832-841

Roth, R.A. and Wallace, K.B. (1980) Disposition of biogenic amines and AI by lungs of SHR. Am. J. Physiol. 239 (6):H736-741.

Roth, B.L., Nakaki, T, Chuang, D.H. and Costa, E. (1984) Aortic regulation sites for 5HT are coupled to phospholipase C and modulate phosphoinositol (PI) turnover. Neuropharmacology 23 (10):1223-1225.

Rubin R.P. and Laychock S.G. (1978) Prostaglandins and calcium membrane interactions in secretory glands. Ann N. Y Academy Sciences. 307:377-390.

Rubin R.P. (1982) Calcium and cellular secretion Chapter 3 and 4. Plenum press. New York and London.

Ryan J.W. (1967) Renin-like enzymes in the adrenal gland. Science 157: 1589-1590.

Saavedra J.M., Brownstein, M. and Jaffe B.M. (1976) A specific and sensitive enzymatic-isotopic microassay for serotonin in tissues. J. Pharmacol. Exp. Ther. 186:508-515.

Sandberg A.A., Nelson D.H., Parker J.G. (1953) The effects of epinephrine on the metabolism of 17-hydroxsteroids in the human. J. Clin. Endocrinol. Metab. 12:629-647.

Sanders-Bush E., Bushing J.A., and Sulser F (1975) Long-term effects of p-Chloroamphetamine and related drugs on central serotonergic mechanisms. J. Pharmacol. Exp. Ther. 192:33-41.

Saruta T. and Kaplan N.M. (1972) Adrenocortical steroidogenesis: Studies on the mechanism of action of AII and electrolytes. J. Clin. Invest. 51:2239-2245.

Sasa C.L., Blank D.C., Wenke D.C. and Sczupak, C.A. (1978) Liquid chromatographic determination of serotonin in serum and plasma. Clin. Chem. 24:1509-1514.

Schiffrin L.E., Chartier L., Thibault G., St-Louis, J., Canten, M. and Genest, J. (1985) Vascular and adrenal receptors for ANF in the rat. Circ. Res. 56:801-807.

Schmidtke J., Wienker T., Flugel, M. and Engel, G. (1976) In vitro inhibition of cyclic AMP phosphodiesterase by cortisol. Nature 262:593-594.

Schulster D (1973) Regulation of steroidogenesis by ACTH in a superfusion system for isolated adrenal cells. Endocrinology 93:700-704.

Schulster D, Schwyzer R. (1980) Cellular receptors for hormones and neurotransmitters. (Schulster D. and Levitzki, A. eds), John Wiley

Semerdjian - Rouquier L. (1981) Determination of 5-hydroxytryptophan, 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in rat and human brain and biological fluids by reversed-phase HPLC with electrochemical detection. J. Chromat 218:663-670.

Sen, S., Valenzuela, R., Smeby, R., Bravo, E.L. and Bumpus, F.M. (1981a) Localization, purification and biological activity of a new aldosterone stimulating factor. Hypertension 3 (Pt. 2) :181-8

Sen S., Shainoff J.R., Bravo E.L. and Bumpus, F.F. (1981b) Isolation of Aldosterone stimulating factor and its effect on adrenal glomerulosa cells in vitro. Hypertension 3(1):4-10.

Sequeira S.J. and McKenna T.J. (1985) Examination of the effects of epinephrine, norepinephrine, and dopamine on aldosterone production in bovine glomerulosa cells in vitro. Endocrinology 117(5):1947-1952.

Shah S.V., Northrup T.E., Hui Y.S.F. and Dousa, T.P. (1979) Action of serotonin on cyclic nucleotides in glomeruli of rat renal cortex. Kidney International 15:463-472.

Shaikh, S., Williams, B.C. and Edwards, C.R.W. (1984) The specificity of ketanserin in the inhibition of the 5HT induced steroidogenic response in the rat zona glomerulosa cells. Abstr. 70. Proc. 10th Scientific Meeting of the International Society of Hypertension, Interlaken, Switzerland.

Shaikh, S., Williams, B.C., Gow, I.F. and Edwards, C.R.W. (1986) The effects of altering endogenous 5HT levels in the rat on the steroidogenic response of zona glomerulosa cells to angiotensin II and 5HT in vitro. Abstr. 90. 174th Meeting of the Society for Endocrinology, London, U.K.

Shenker Y., Gross M.D. and Grekin R.J. (1985) Central serotonergic stimulation of aldosterone secretion. J. Clin. Invest. 76:1485-1490.

Shenker Y., Villareal J.Z., Sider R.S. and Grekin, R.G. (1985) α - melanocyte hormone stimulation of aldosterone secretion in hypophysectomised rats. Endocrinology 116(1):138-141.

Shima S., Komoriyama K., Hirai M. (1978) Studies on cyclic nucleotides in the adrenal gland : VIII Effects of AII on adenosine 3', 5' monophosphate and steroidogenesis in the adrenal cortex. Endocrinology 103(4):1361-1367.

Shima S., Komoriyama K., Hirai M., and Asakura, M. (1980) Studies on cyclic nucleotides in the adrenal gland X: Effects of ACTH and prostaglandins on adenylate cyclase in the adrenal cortex. Endocrinology 106:948-951.

Shima S., Komoriyama K., Hirai, M. and Koyama, H. (1984) Studies on cyclic nucleotides in the adrenal gland XI: Adrenergic regulation of adenylate cyclase in the adrenal cortex. Endocrinology 114(2):325-329.

Shenker Y., Gross M.D. and Grekin R.J. (1985) Central serotonergic stimulation of aldosterone secretion J. Clin. Invest. 76:1485-1490.

Sheppard H., Swenson R. and Mowles T.F. (1963) Steroid biosynthesis by rat adrenal: functional zonation. Endocrinology 73:819-824.

Simonian, M.H., Hornsby P.J. and Ill C.R. (1979) Characterisation of cultured bovine adrenal cortical cells and derived clonal lines: regulation of steroidogenesis and culture life span. Endocrinology 105:99-108.

Simonian, M.H., White M.L. and Gill G.N. (1982) Growth and function of cultured bovine adrenocortical cells in serum-free defined medium. Endocrinology 111:919-927.

Simonian, M H., and Capp M.W. (1984) Characterisation of steroidogenesis in cell cultures of the human foetal adrenal cortex: comparison of definitive and foetal zones. J. Clin. Endocrinol. Metab. 59:643-651.

Smythe, G.A. (1979) Serotonin in 'Hormones in Blood', pp209-236, (Gray, C.H. and James, V.H. eds), Academic Press.

Snow,, P.J.D., Lennard-Jones, J.E., Curzon, G. and Stacey, R.S. (1955) Humoral effects of metastasising carcinoid tumours. Lancet 269:1004-1009.

Snyder S.H., Axelrod J. and Zweig M. (1965) A sensitive and specific fluorescence assay for tissue serotonin. Biochem. Pharmacol. 14:831-835.

Sowers, J.R., Tuck M.L., Golub M.S. and Solavs, E.G. (1980) Dopaminergic modulation of aldosterone secretion is independent of alterations in renin secretion. *Endocrinology* 107(4):937-941.

Sowers, J.R., Brickman A.S., Sowers D.K. and Berg, G. (1981) Dopaminergic modulation of aldosterone secretion is unaffected by glucocorticoids and AII blockade *J. Clin. Endocrinol. Metab.* 52(6):1078-1084.

Spat, A. and Jozan, S. (1975) Effect of prostaglandin E_2 and A_2 on steroid synthesis by rat adrenal gland. *J. Endocrinol* 65:55-63.

Spat, A., Antoni, F.A. and Nagy, K. (1977) Effect of prostaglandins synthetase inhibitors on basal and ACTH stimulated steroidogenesis by seperated adrenal cortical zones. *J. Steroid Biochem.* 8:293-298.

Steinbusch, H.W.M., Verhofstad, A.A.J. and Hoosten, H.J.W. (1978) Localisation of serotonin in the central nervous system by immunohistochemistry: description of a specific and sensitive technique and some applications. *Neuroscience* 3:811-819.

Stern, N., Ozaki, L., and Tuck, M.L. (1986) Evidence for dopaminergic binding sites in human adrenal cortex. *Metabolism* 35(12):1154-1158.

Stollak, J.S. and Furchgot, R.F. (1982) Use of selective antagonists for determing the types of receptors mediating the actions of 5-hydroxytryptamine in the isolated rabbit aorta. *J. Pharmacol. Exp. Ther.* 224(1):215-220.

Swanson, L.W. and Sawchenko, P.E. (1980) Paraventricular nucleus: a site for the integration of neuroendocrine and autonomic mechanisms. *Neuroendocrinology* 31:410-417.

Sweat, M.L. and Bryson, M.J. (1965) Steroid 11- β -oxygenation, ascorbic acid, epinephrine and adrenochrome interrelations in the adrenal gland. *Endocrinology* 76:773-775.

Symington, T. (1962) Morphology and secretory cytology of the human adrenal cortex. *Br. Med. Bull* 18:117-121.

Tait, S.A.S., Schulster, D., Okamoto, M., Flood, and Tait, J.F. (1970) Production of steroids by in vitro superfusion of endocrine tissue II Steroid output from bisected whole capsular and decapsulated adrenals of normal intact, hypophysectomised and hypophysectomised-nephrectomised rats as a function of time of superfusion. *Endocrinology* 86:360-382.

Tait, S.A.S., Tait, J.F. and Bradley, J.E.S. (1972) The effect of 5HT and potassium on corticosterone and aldosterone production by isolated zona glomerulosa cells of the rat adrenal cortex. Aust. J. Exp. Bio. Med. Sci. 50:833-846.

Tait, S.A.S., Tait, J.F. Gould, R.P., Brown, B.L. and Albano, J.D. (1974) The preparation and use of purified and unpurified dispersed adrenal cells and a study of the relationship of their cAMP and steroid output. J Steroid Biochem. 5:775-787.

Tait, J.F., Tait, S.A.S., Albano, J.D.M., et al., (1975) The response of purified zona glomerulosa cells of the rat adrenal to stimulation by K⁺, serotonin, ACTH, AII and cAMP, pp19-24, in Research of Steroids, (Breuer, H., Hughes, A., Kloppe, A., Conti, C., Jungblut, F. and Lerner, L. eds), North-Holland

Tait, J.F. and Tait, S.A.S. (1976) The effect of changes in potassium concentration on the maximal steroidogenic response of purified zona glomerulosa cells to angiotensin II. J. Steroid Biochem. 7:687-690.

Tait, J.F., Tait, S.A.S., Bell, J.B., Hyatt, P.J. and Williams, B.C. (1980a) Further studies on the stimulation of rat adrenal capsular cells. Four types of response. J. Endocrinol. 87:11-27.

Tait, J.F., Tait, S.A.S. and Bell, J.G. (1980b) Steroid hormone production by mammalian adrenocortical dispersed cells. Essays in Biochem 16:99-174.

Takai, Y., Kikkawa, U., Kaibuchi, K. and Nishizuka, Y. (1985) Membrane phospholipid metabolism and signal transduction for protein phosphorylation: 137-145 in Advances in Cyclic Nucleotide and Protein Phosphorylation Research 18, (Greengard, P. and Robison, A. eds), Raven Press.

Taleisnik, S., Tomatis, M.E., Celis, M. (1972) Role of catecholamines in the control of melanocyte-stimulating hormone secretions in rats. Neuroendocrinology 10:235-45.

Tamir, H., Bebirian, R., Muller, F. and Casper, D. (1980) Differences between intracellular platelet and brain proteins that bind serotonin. J Neurochem 35(5):1033-1044.

Thoa, N.B., Eccleston, D. and Axelrod, J. (1969) The accumulation of [¹⁴C]-5HT in the guinea pig vas deferens. J. Pharmacol. Exp. Ther 20:873-876.

Tipton, K.F., Houslay, M.D. and Mantle, T.J. (1976) The nature and localisation of the multiple forms of monoamine oxidase from Monoamine Oxidase and its inhibition. A Ciba Foundation Symposium, Elsevier, Excerpta Medica and North Holland.

Titeler, M., Battaglia, G. and Shannon, M. (1984) Guanine nucleotides modulate cortical S2 5HT receptors. J. Rec. Res. 4(7):705-712.

Torda, T. and Kvetnansky, R. (1983) Effect of adrenal medullectomy on the activity of COMT and MAO in the adrenal cortex and stress in rats. Endocrinol. Experimenta 17:47-52.

Trost, B.N., Muller, J. (1976) Uptake and metabolism of serotonin by rat adrenal tissue in vitro. Acta Endocrinol. 82:353-365.

Udenfriend, S., Clark, C.T., Titus, E. (1953) Hydroxylation of the 5-position of tryptophan as the first step in the metabolic conversion to serotonin. Fed. Proc. 12 :282 Abstr 932.

Ulick, S. (1976) Diagnosis and nomenclature of the disorders of the terminal part of the aldosterone biosynthetic pathway. J. Clin. Endocrinol. Metab. 43:92-98.

Uno, H. (1977) Catecholaminergic terminals in the perisinusoidal spaces of the hepatic acini and adrenal cortex of the macaques. Anat Rec. 187:735.

Unsicker, K. (1971) On the innervation of the rat and pig adrenal cortex. Z. Zelforsch 116:151-156.

Vaccari, A., Brothman, S., Cimino, J. and Timiras, P.S. (1977) Sex differentiation of neurotransmitter enzymes in central and peripheral nervous systems. Brain Res. 132(1): 176-185.

Van de Kar, L.D., Kartesz, M., Bethea, C.L. and Ganong, W.F. (1985) Serotonergic stimulation of prolactin and corticosterone secretion is mediated by different pathways from the mediobasal hypothalamus. Neuroendocrinology 41:380-384.

Van Loom, G.R. and Sole, M.J. (1980) Plasma dopamine: source, regulation and significance. Metabolism 11 (Supp 1):1119-1123.

Van Neuten, J.M., Janssen P.A.J., Beek J.V., Xhonneux, R., Verbeuren, T.J. and Vanhoutte, P.M. (1981) Vascular effects of ketanserin (R 41 468), a novel antagonist of 5HT₂ serotonergic receptors. J. Pharm. Exp. Ther. 218(1):217-230.

Van Neuten, J.M., Janssen, P.J., De Ridder, W. and Vanhoutte, P.M. (1982a) Interaction between 5-Hydroxytryptamine and other vasoconstrictor substances in the isolated femoral artery of the rabbit: effect of ketanserin (R 41 468). Eur. J. Pharmacol. 77:281-287.

Van Neuten, J.M., Leysen, J.E., Vanhoutte, P.M. and Janssen, P.A.J. (1982b): Serotonergic responses in vascular and non-vascular tissue. Arch Inst Pharmacodyn Ther 256:331-334.

Van Neuten J.M. (1983) 5-hydroxytryptamine and precapillary vessels. Fed. Proc. 42(2):223-227.

Vanable W.J. (Jr) (1963) A ninhydrin reaction giving a sensitive quantitative fluorescence assay for 5HT. Anal. Biochem. 6:393-403.

Vanhoutte P.M. (1985) Can peripheral serotonergic blockade explain the hypotensive effect of ketanserin? J. Cardiovasc. Pharmacol. 7(Supp 17) S105-S109.

Vanhoutte P.M. (1982) Does 5-hydroxytryptamine play a role in hypertension? Tr. Pharmacol. Sci.: 370-373.

Varndell, I.M. Pollak, J.M., Allen, J.M., Terenghi, G. and Brown, S.M. (1983) Neuropeptide tyrosine (NPY) immunoreactivity in norepinephrine containing cells and nerves of mammalian adrenal gland. Endocrinology 114:1460-1461.

Verhofstadt, A.A.J., and Jonsson, G. (1983) Immunohistochemical and neurochemical evidence for the presence of serotonin in the adrenal medulla of the rat. Neuroscience 10(4):1443-1453.

Vernikos-Danellis, J., Berger, P. and Barchas, J.D. (1973) Brain serotonin and pituitary-adrenal function. Prog Brain Res 39:301-309.

Viveros, O.H., Diliberto, E.J., Jr. and Hazim, E. (1979) Opiate-like materials in the adrenal medulla: evidence for storage and secretion with catecholamines. Mol. Pharmacol. 16:1101-1108.

Vinson, G. P. and Whitehouse, B.J. (1969) The relationship between steroid production from endogenous precursors and from added radioactive precursors by rat adrenal tissue in vitro. The effects of corticotrophin. Acta Endocrinol. 61:695-708.

Vinson, G.P. and Whitehouse, B.J. (1973) The biosynthesis and secretion of aldosterone by the rat adrenal zona glomerulosa and the significance of the compartmental arrangement of steroids. Acta Endocrinol. 72:746-752.

Vinson, G.P. Braysher, M. and Whitehouse, B.J. (1975) In vitro steroidogenesis by the non-zonal adrenal tissue of the skink, Tiliqua rugosa. Gen. Comp. Endocrinol. 26:541-549.

Vinson, G.P. and Whitehouse, B.J. (1976) In vitro modification of rat adrenal zona fasciculata/reticularis function by the zona glomerulosa. Acta Endocrinol. 81:340-350.

Vinson, G.P., Whitehouse, B.J., Goddard, C. and Sibley, C.P. (1979) Comparative and evolutionary aspects of aldosterone secretion and zona glomerulosa function. J. Endocrinol. 81:5P-24P.

Vinson, G.P., Whitehouse, B.J., Dell, A., Etienne, T. and Morris, H.R. (1980) Characterisation of an adreanal zona glomerulosa stimulating component of posterior pituitary extracts as α -MSH. *Nature* 284:464-467.

Vinson, G. P., Whitehouse, B.J., Dell, A., Etienne, A.T. and Morise, H.R. (1981) Specific stimulation of steroidogenesis in rat adrenal zona glomerulosa cells by pituitary peptides. *Biochem. Biophys. Res. Commun.* 99 (1):65-72.

Vinson, G.P., Whitehouse, B.J., Dell, A., Bateman, A. and McAnley, M.E. (1983) α MSH and zona glomerulosa function in the rat. *J. Steroid Biochem.* 19(1):537-544.

Vinson, G.P., Whitehouse, B.J., Bateman, A., Hruby, K.J., Sawyer, T.K. and Darman, P.S. (1984) Alpha MSH analogues and adrenal zona glomerulosa function. *Life Sci.* 35:603-610.

Vinson, G.P., Hinson, J.P. and Raven, P.W. (1985) The relationship between tissue preparation and function; A method for the study of control of aldosterone and secretion: A review. *Cell Biochemistry and Function* 3:235-253.

Viveros, O.H., Diliberto, E.J., Jr. and Hazim, E. (1979) Opiate-like materials in the adrenal medulla: evidence for storage and secretion with catecholamines. *Mol. Pharmacol.* 16:1101-1108.

Vogt, M. (1944) The effect of chronic administration of adrenaline in the suprarenal cortex and the comparison of this effect with that to hexestrol. *J. Physiol. (Lond)* 104:60-70.

Welsh, J.H. (1968) Distribution of 5HT in nervous systems of various animal species. *Adv. Pharmacol.* 6A:171-188.

Wenting, G.J., Woittiez, A.J., Man in't Veld, A.J. and Shalekamp, M.A.D.H. (1984) Serotonin, alpha adrenoreceptors and blood pressure. Effects of ketanserin in essential hypertension and autonomic insufficiency. *Hypertension* 6(1):100-109.

Whitfield, L., Sowers, J.R., Tuck, M.L. and Golub, M.S. (1980) Dopaminergic control of plasma catecholamines and aldosterone response to acute stimuli in normal man. *J. Clin. Endocrinol. Metab.* 51(4):724-729.

Whitehouse, B.J.W. and Vinson, G.P. (1971) Compartmental arrangement of steroid precursors and the control of steroid hormone secretion in the rat adrenal tissue in vitro. *Acta Endocrinol. (Copenh)* 68:467-476.

Whitehouse, B.J. and Vinson, G.P. (1973) Compartmental arrangement of steroids formed from ^{14}C acetate by rat adrenal zona glomerulosa and the effect of corticotrophin. *Acta Endocrinol (Copenh)* 72:737-745.

Whitehouse, B.J. and Vinson, G.P. (1975) Formation of corticosteroids in vitro by interrenal tissue from the teleost fish, *coregonus clupeoides*. Gen Comp. Endocrinol. 27:305-313.

Whitehouse, B.J., Vinson, G.P. and Thody, A.J. (1982) Dopaminergic control aldosterone: modulation of the response of rat adrenal zona glomerulosa cells to α -MSH by pre-treatment with bromocriptine or metoclopramide. Steroids 39(2):155-164.

Wilcox, C.S., Aminoff, M.J., Millar, J.G.B. Keenan, J. and Kremer, M. (1975) Circulating levels of corticotrophin and cortisol after infusions of L-dopa, dopamine and norepinephrine in Man. Clin. Endocrinol. 4:191-198.

Williams, G.H., Rose, L.I., Dluhy, R.G., Dingman, J.F. and Lauer, D.P. (1971) Aldosterone response to sodium restriction and ACTH stimulation in panhypopituitarism. J. Clin. Endocrinol. 32:27-34.

Williams, G.H., McDonnell, L.H., Tait, S.A.S. and Tait, J.F. (1972) The effect of medium composition and in vitro stimuli on the conversion of corticosterone to aldosterone in the rat glomerulosa tissue. Endocrinology 91:948-60.

Williams, B.C., McDougall, J.G., Tait, J.F. and Tait, S.A.S. (1981) Calcium efflux from superfused isolated rat adrenal glomerulosa cells. Clin. Sci. 61:541-555.

Williams, B.C., Shaikh, S. and Edwards, C.R.W. (1984) The specificity of the ketanserin in the inhibition of serotonin-induced steroidogenesis in the rat adrenal zona glomerulosa. J. Hypertens. 2 (Suppl 3):559-561.

Wilson, T.A., Keiser, D.L. and Carey, R.M. (1983) Dopamine inhibits aldosterone secretion in Man is independent of the autonomic system. J. Clin. Endocrinol. Metab. 57:200-203.

Winkler, H., Apps, D.K. and Fischer-Colbrie, R. (1986) The molecular function of adrenal chromaffin granules: established facts and unresolved topics. Neuroscience 18 (2):261-290.

Wright, N.A. (1971) Cell proliferation in the pre-pubertal male rat adrenal cortex: An in vitro study. J. Endocrinol. 49 :599-609.

Wright, N.A., Voncina, D. and Morlem, A.R. (1973) An attempt to demonstrate cell migration from the zona glomerulosa in the pre-pubertal male rat adrenal cortex. J. Endocrinol. 59:451-459.

Wright, N.A., Appleton, D.R. and Hornsby, A.R. (1974) Effect of dexamethasone on cell population kinetics in the adrenal cortex of the prepubertal male rat. J. Endocrinol. 62:527-536.

Wurtman, R.J. and Axlerod, J. (1966) Control of enzymatic synthesis of adrenals in the adrenal medulla by adrenal cortical steroids. J. Biol Chem. 241(10):230-12305.

Yamakado, M., Franco-Saenz, R. and Mulrow, P.J. (1982) α -MSH stimulation of aldosterone production: effect of low salt diet and site of action. Abstr. 224 64th Ann. Meeting Endocrinol. Soc. (San Francisco, USA).

Yamanako, K., Kigoshi, S. and Muramatsu, I. (1986) Muscarinic receptor subtypes in bovine adrenal medulla. Biochem. Pharmacol. 35 (18):3151-3157.

Youdim, M.B.H. and Holzbauer, M. (1976) Physiological aspects of the oxidative deamination of monoamines in Symposium on Monoamine Oxidase and its Inhibition. Ciba Foundation Symp 39:105-135, Elsevier Excerpta Medica.

Zager, P.G., Luetscher, J.A. (1982) Effects of AIII and ACTH on aldosterone secretion. Clin. Exp. Hypertens. Theory and Practice A4(9+10):1481-1504.

Zern, R.T., Bird, J.L. and Feldman, J.M. (1980) Effect of increased pancreatic islet norepinephrine, dopamine and serotonin concentration on insulin secretion in the golden hamster. Diabetologia 18:341-346.

Zimmerman, H. and Ganong, W.F. (1980) Pharmacological evidence that stimulation of central serotonergic pathways increase renin secretion. Neuroendocrinology 30(2):101-107.